IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants	:	Rattenholl *et al.*	)	
			)	Group Art Unit: 1649
Appln. No.	:	09/807,096	)	
			)	Examiner: Robert Clinton Hayes
Filed	:	November 19, 2001	)	

For : METHOD FOR OBTAINING ACTIVE BETA-NGF

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APPEAL BRIEF UNDER 37 C.F.R. § 41.37

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal pursuant to 35 U.S.C. § 134 from the Examiner's decision rejecting claims 8, 20, and 26-29, as set forth in the Final Official Action dated January 15, 2010.

I. Real Party in Interest

The real party in interest is Scil Proteins GmbH (hereinafter "Scil"), a corporation duly organized under the laws of the country of Germany and the assignee of the inventors' entire interest by virtue of an assignment from the co-inventors to Scil that

was recorded at Reel 019168, Frame 0432, in the assignment Division of the United States Patent and Trademark Office (hereinafter the "Patent Office") on April 16, 2007.

II. Related Appeals and Interferences

There are no appeals or interferences known to appellants or appellants' legal representatives that will directly affect or be directly affected by or have a bearing on the decision of the Board of Patent Appeals and Interferences (hereinafter "the Board") in the pending appeal.

III. Status of Claims

Claims 8, 20, and 26-29 are pending in the subject application. Claims 1-7, 9-19, and 21-25 have been canceled by appellants. Claims 8, 20, and 26-29 stand finally rejected as per a Final Official Action dated January 15, 2010 (hereinafter the "Final Official Action"), and are the subject of this Appeal.

IV. Status of Amendments

There are no pending amendments known to appellants or appellants' legal representatives.

V. Summary of Claimed Subject Matter

This summary is presented pursuant to the requirements of 37 C.F.R. § 41.37(c)(1)(v) mandating a "concise explanation of the subject matter defined in each of the independent claims involved in the appeal". Nothing stated within this summary is to

be interpreted as changing the specific language of the claims, nor is the language of this summary intended to be construed so as to limit the scope of the claims in any way.

Independent claim 8 recites a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient (original claims 8 and 9), wherein the purified human proNGF is purified to at least 90% purity (page 16, first full paragraph (inclusion bodies "always contained approx. 90-95% rh proNGF"); and Figures 2, 4, 6, and 7 in view of Examples 3 and 4), has an activity in vivo analogous to β-NGF (page 4, line 33, through page 5, line 1), and promotes survival of dorsal root ganglia (DRG) sensory neurons (page 22, line 15, through page 23, line 3).

Stated another way, independent claim 8 recites a pharmaceutical composition that comprises (i) human proNGF as the active ingredient, wherein the purified human proNGF is purified to at least 90% purity, has an activity in vivo analogous to β-NGF, and promotes survival of dorsal root ganglia (DRG) sensory neurons; and (ii) a pharmaceutically acceptable carrier.

Independent claim 29 recites a pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient (original claims 8 and 9), wherein the purified human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis (page 22, line 15, through page 23, line 3).

Thus, independent claim 29 recites a pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the activity of the purified human proNGF is claimed relative to that of mature human β-NGF in a standard DRG assay.

Summarily, independent claims 8 and 29 recite pharmaceutical preparations that comprise purified human proNGF as the active ingredient.

VI. Grounds of Rejection to be Reviewed on Appeal

The grounds of rejection for review are as follows:

(A) The rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 102(b) as being anticipated by U.S. Patent No. 5,683,894 to Edwards *et al.* (hereinafter "Edwards");

(B) The rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,169,762 to Gray (hereinafter "Gray") and U.S. Patent No. 5,235,043 to Collins *et al.* (hereinafter "Collins"); and

(C) The rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 103(a) as being unpatentable over Gray and/or Collins and/or Japanese Patent Application No. JP 09-023883 of Boehringer Mannheim GmbH/Lang *et al.* (hereinafter "JP 09-023883").

VII. Arguments

A. Rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 102(b) as being anticipated by U.S. Patent No. 5,683,894 to Edwards

A.1. Argument for independent claim 8

The rejection of claim 1 as anticipated by Edwards should be reversed because Edwards does not teach each and every element of the claim. Particularly, appellants

respectfully submit that Edwards fails to disclose (i) any compositions that one of ordinary skill in the art would reasonably consider to be pharmaceutical preparations of human proNGF; (ii) any preparation in which the purified human proNGF is present as the active ingredient; (iii) any preparation in which the purified human proNGF is purified to at least 90% purity; and (iv) any preparation in which the purified human proNGF has an activity in vivo analogous to β-NGF and promotes survival of dorsal root ganglion (DRG) sensory neurons.

A.1.a. Edwards does not disclose pharmaceutical preparations of human proNGF

In support of the instant rejection, the Patent Office asserted, "Edwards et al teach how to make a pharmaceutical composition comprising a recombinant pro-NGF solution (e.g., cols. 5 & 7-9), which can also be 'derived from humans' (e.g., col. 4, lines 40-42), which inherently comprises SEQ ID NO: 4 and inherently is encoded by a nucleic acid comprising SEQ ID NO: 3" (*see* Final Official Action dated January 15, 2010 (hereinafter the "Final Official Action") at pages 3-4). The Patent Office further asserted:

> Edwards clearly teach recombinant "NGF-beta... administered as a pharmaceutical composition..." (col. 5, lines 57-58). See col. 5, lines 49-col. 6, line 14). Edwards also teach "one may cleave and activate the pro-NGF-beta to the mature form either before *or after isolation from the expression host* [emphasis added]"; thereby, demonstrating disclosure of an isolated pro-NGF-beta solution (col. 5, lines 17-19). Example 5 (in column 5) discloses "pro-NGF-beta *prepared in vitro* as described in Example 2 above was substituted for pro-NGF-beta prepared *in vivo*" (col. 8, lines 38-40 & 44-46). Column 8 (lines 60) discloses "[p]ro-NGF-beta *purified* from mouse L929... [emphases added]". Column 9 discloses expression of "pro-NGF-beta in yeast for large scale fermentation" (col. 9, lines 16-39), Example 2 (in column 7) discloses preparation of "mouse pro-NGF-beta using an in vitro expression system, for comparison with active NGF-beta..." (col. 7, line 7-8). Simply put, as long as Edwards

> teach their pro-NGF-beta solution that comprises a pharmaceutically-acceptable carrier water, etc., the limitation of a pharmaceutical preparation are met; especially when claim 8 recites the open claim language of "[a] pharmaceutical composition *comprising*...".

Non-Final Official Action dated June 17, 2009 at page 4 (emphases supplied).

Initially, appellants respectfully submit that the only discussions of pharmaceutical compositions in Edwards relate to pharmaceutical compositions of mature NGF-β. This can be seen at col. 5, line 49, through col. 6, line 29 of Edwards, which disclose administration of NGF-β ("NGF-beta may be administered as a pharmaceutical composition comprising mature, active NGF-beta in combination with a pharmaceutically acceptable excipient"; *see* Edwards at col. 5, lines 57-59); encapsulated NGF-β ("one may incorporate or encapsulate NGF-beta in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally"; *see* Edwards at col. 6, lines 3-7); lyophilized preparations of NGF-β ("one may provide NGF-beta in solid form, especially as a lyophilized powder"; *see* Edwards at col. 6, lines 8-10); and amounts of NGF-β that are required to treat particular neural disorders (*see* Edwards at col. 6, lines 15-29). Additional references to pharmaceutical preparations can be found in Example 8 of Edwards, which discloses injectable formulations of NGF-β and NGF-β "depot" formulations. Appellants respectfully submit that Edwards defines "NGF-β" to be "the pure, active, mature beta subunit of 7S NGF" (*see* Edwards at col. 3, lines 33-34). Therefore, appellants respectfully submit that each and every reference to a pharmaceutical preparation in Edwards relates to preparations of pure, active mature NGF-β, and not proNGF. For this additional reason, appellants respectfully submit that

Edwards does not disclose any pharmaceutical preparations of human proNGF as recited in instant claim 8.

Furthermore, the Patent Office's assertion that Edwards teaches that "one may cleave and activate the pro-NGF-beta to the mature form either before *or after isolation from the expression host* [emphasis added]"; thereby, demonstrating disclosure of an isolated pro-NGF-beta solution" fails to support the instant rejection. Appellants respectfully submit that even assuming *arguendo* that Edwards discloses "an isolated pro-NGF-beta solution", there is no disclosure in Edwards that such a solution is a pharmaceutical preparation. Appellants respectfully submit that there is no basis for the Patent Office to conclude that "isolated pro-NGF-beta solution" is synonymous with "pharmaceutical preparation of human proNGF", and thus the Patent Office's apparent attempt to equate these phrases is believed to be improper.

Continuing, appellants respectfully submit that the Court of Appeals for the Federal Circuit (CAFC) has stated that "[d]uring reexamination, as with original examination, the PTO must give claims their broadest reasonable construction consistent with the specification" (*see In re Suitco Surface, Inc.*, 603 F.3d 1255, 1259 (Fed. Cir. 2010); emphases added), and that "claim language should be read in light of the specification as it would be interpreted by one of ordinary skill in the art." *In re Bond*, 910 F.2d 831, 833 (Fed. Cir. 1990), *quoting In re Sneed*, 710 F.2d 1544, 1548 (Fed. Cir. 1983); emphasis added. Appellants respectfully submit that the phrase "pharmaceutical composition" would be understood by one of ordinary skill in the art to be a composition formulated for administration to a subject. As such, appellants respectfully submit that this understanding would not only inform one of ordinary skill in the art with respect to

what might be acceptable for inclusion in a pharmaceutical preparation, but it would also provide a basis for what one of ordinary skill in the art would not include.

For example, appellants have argued that each composition disclosed in Edwards include at least one component that would lead one of ordinary skill in the art to exclude the composition from any reasonable definition of "pharmaceutical preparation". For example, the Patent Office argued that the *in vitro* translated preparations of murine proNGF would properly be considered "pharmaceutical preparations". Appellants respectfully submit, however, that the products of *in vitro* translation reactions are contaminated with the myriad proteins, nucleic acids (*e.g.*, ribosomal RNAs, transfer RNAs, etc.), amino acids, and other biomolecules that are present in the translation reaction itself. In the specific case of Edwards, the *in vitro* translations were performed using a wheat germ extract (*see* Edwards at col. 7, lines 21-23). Thus, appellants respectfully submit that one of ordinary skill in the art would understand that the *in vitro* translated products disclosed in Edwards would be contaminated with numerous wheat germ proteins, and as such, would not be understood to be a "pharmaceutical preparation".

Additionally, the *in vitro* translations disclosed in Edwards employed ^{35}S-methionine as a label. Appellants respectfully submit that one of ordinary skill in the art would understand that ^{35}S-methionine, a radioactive isotope of sulfur, would not be employed in a pharmaceutical preparation. The Patent Office argued, however, that "a pharmaceutical composition could also contain ^{35}S-methionine", especially when one wants to image, detect, etc. whether their 'mouse pro-NGF-beta' became bound to the appropriate cells" (*see* Final Official Action at page 5). Appellants respectfully submit,

however, that one of ordinary skill in the art would also know that the disintegration product of ^{35}S is too weak to be used for *in vivo* imaging and/or detection, and thus the Patent Office's mere speculation as to why someone *might* want to incorporate ^{35}S into a pharmaceutical preparation is factually inaccurate and fails to support the instant rejection.

The remaining assertions by the Patent Office reproduced hereinabove are also believed to fall far short of that required to support a rejection of claim 8 under 35 U.S.C. § 102(b). For example, the Patent Office asserted that "Edwards also teach 'one may cleave and activate the pro-NGF-beta to the mature form either before *or after isolation from the expression host*'; thereby, demonstrating disclosure of an isolated pro-NGF-beta solution (col. 5, lines 17-19)" *see* Final Official Action at page 4; emphases supplied). As such, it appears that the Patent Office based the instant assertion on an inherency theory, in which the Patent Office appears to contend that an isolated pro-NGF-beta solution would inherently be a pharmaceutical preparation.

Appellants respectfully traverse this apparent assertion. Appellants respectfully submit that the mere possibility that an isolated preparation *might* be (or *could* be) formulated as a pharmaceutical is not sufficient to satisfy the requirements of inherent disclosure. Particularly, appellants respectfully submit that the CAFC has made clear that inherency "may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient"; *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999); emphases added). *See also* M.P.E.P. § 2163.07(a). With respect to the instant rejection, appellants respectfully submit that even assuming *arguendo* that Edwards disclosed an

isolated pro-NGF-beta solution", instant claim 8 is directed to pharmaceutical preparations of human proNGF. There is believed to be no support for the Patent Office's apparent contention that an "isolated pro-NGF-beta solution" would necessarily be a pharmaceutical preparation of human proNGF as recited in instant claim 8. Thus, appellants respectfully submit that this assertion also fails to support the instant rejection of claim 8 under 35 U.S.C. § 102(b).

Next, the Patent Office asserted that "Example 5 (in column 5 [sic]) discloses "pro-NGF-beta *prepared in vitro* as described in Example 2 above was substituted for pro-NGF-beta prepared *in vivo*" (col. 8, lines 38-40 & 44-46)" (*see* Final Official Action at page 4). Similar to the above, appellants respectfully submit that as would be understood by one of ordinary skill in the art, neither pro-NGF-beta prepared *in vitro* or *in vivo* would necessarily be a pharmaceutical preparation of human proNGF as recited in instant claim 8. Thus, appellants respectfully submit that the instant assertion does not support the rejection of claim 8 under 35 U.S.C. § 102(b).

Additionally, the proNGF preparations described in Examples 2 and 5 are preparations of murine proNGF, not human NGF. *See* Example 2 of Edwards at column 7 ("This preparation describes the expression of mouse pro-NGF-beta using an in vitro expression system) and Example 5 of Edwards at column 8 ("pro-NGF-beta prepared in vitro as described in Example 2 above was substituted for pro-NGF-beta prepared in vivo"). The Patent Office asserts that "Column 8 (lines 60) discloses "[p]ro-NGF-beta *purified* from mouse L929... [emphases added]". Column 9 discloses expression of "pro-NGF-beta in yeast for large scale fermentation" (col. 9, lines 16-39), [and] Example 2 (in column 7) discloses preparation of "mouse pro-NGF-beta using an in vitro

expression system, for comparison with active NGF-beta..." (col. 7, line 7-8)" (*see* Final Official Action at page 4). As such, appellants respectfully submit that in each instance, the "pro-NGF-beta" described is murine proNGF. With respect to column 8, line 60, Edwards discloses that "[p]ro-NGF-beta was purified from mouse L929 cells infected with W:NGF-A and W:NGF-B" and digested with trypsin, which one of ordinary skill in the art would recognize was to activate the inactive pro-NGF-beta to the fully active mature NGF-β. "W:NGF-A" and "W:NGF-B" are viral constructs that express murine pro-NGF-beta as set forth in Example 3 of Edwards. As set forth therein, the NGF-beta cDNA "as prepared in Example 1" was employed for generating this viral constructs (*see* Edwards at col. 7, lines 40-41). Example 1 describes cloning of a murine NGF-beta cDNA from "male mouse submaxillary gland" (*see* Edwards at col. 6, lines 37-38).

Therefore, because the passages relied on by the Patent Office fail to disclose pharmaceutical preparations of human proNGF, appellants respectfully submit that the Patent Office's assertions with respect to the Edwards reference do not support a rejection of claim 8 under 35 U.S.C. § 102(b).

Summarily, appellants respectfully submit that there is no disclosure in Edwards that one of ordinary skill in the art would reasonably consider to relate to pharmaceutical preparations of human proNGF as recited in independent claim 8. Rather, appellants respectfully submit that Edwards discloses only preparations of murine proNGF and NGF-β. There is also no disclosure in Edwards of any form of proNGF other than to be used as a starting material to be cleaved to form compositions comprising mature NGF-β. As a result, appellants respectfully submit that Edwards does not disclose each and

every element of independent claim 8, and thus does not support a rejection of claim 8 under 35 U.S.C. § 102(b).

Accordingly, appellants respectfully submit that the Patent Office has not presented a *prima facie* case of anticipation of claim 8 over Edwards. Thus, appellants respectfully request that the instant rejection be reversed.

A.1.b. Edwards does not disclose any preparation wherein human proNGF is present as an active ingredient

Independent claim 8 also recites *inter alia* a pharmaceutical preparation comprising... purified human proNGF as the active ingredient. Appellants respectfully submit that in the context of the claims and after review of the specification, one of ordinary skill in the art would understand that the phrase "human proNGF as the active ingredient" would refer to human proNGF *per se* having "an activity in vivo analogous to β-NGF that promotes survival of dorsal root ganglia (DRG) sensory neurons" (*see* claim 8).

Appellants respectfully submit that the Patent Office has failed to identify any disclosure in Edwards of any proNGF preparation that is biologically active. In fact, and contrary to the Patent Office's assertions, the express disclosure of Edwards states that the murine proNGF preparations tested therein had "little or no activity" (*see* Edwards at col. 9, lines 6-9). Therefore, appellants respectfully submit that there is no disclosure in Edwards that teaches the subject matter of independent claim 8.

The Patent Office relied, however, on an inherency theory for asserting that the Edwards compositions inherently possess the activities of the presently claimed pharmaceutical compositions. According to the Patent Office,

> it has been established by the courts that a product (i.e., the proNGF **product**) inherently possesses characteristics of that product (i.e., possesses any activity inherent to the protein which is derived from its amino acid sequence), and that "the PTO can require an applicant to prove that the prior art products do not necessarily or inherently possess the characteristics of his claimed product". *Ex parte Gray*, 10 USPQ2d 1922 (1989); *In re Best*, 195 USPQ 430 (CCPA 1976).

Final Official Action at page 3 (emphasis supplied). Appellants respectfully submit, however, that even assuming *arguendo* that "the PTO can require an applicant to prove that the prior art products do not necessarily or inherently possess the characteristics of his claimed product", the Edwards reference itself already provides the necessary proof. As set forth hereinabove, Edwards states that the murine proNGF preparations tested had "little or no activity in the DRG assay" (*see* Example 6 of Edwards at col. 9, lines 6-9). The human proNGF of the pharmaceutical preparations of present claim 8 are disclosed in the instant application to have activity that was analogous to mature β-NGF. Thus, appellants respectfully submit that there is evidence already on the record that provides sufficient evidence to demonstrate that "the prior products" (*i.e.*, the murine pro-NGF-beta of Edwards) do not "necessarily or inherently possess the characteristics of [the] claimed product" (*i.e.*, the human proNGF of claim 8) to thereby satisfy appellants' burden under the test set out in *Ex parte Gray*.

Furthermore, appellants respectfully submit that the Patent Office's assertion that the activity of proNGF is inherent from its structural characteristics (presumably referring to its primary amino acid structure) is also explicitly contradicted by the disclosure of Edwards. First, appellants respectfully submit that Edwards discloses in Example 6 that two forms of proNGF had "little or no activity" in the DRG assay, whereas digestion of either of these forms with trypsin resulted in "substantial NGF-β activity". Therefore, the

proNGF produced by the mouse L929 cells as set forth in Example 6, despite presumably having the proper primary amino acid sequence of murine proNGF, was non-functional.

Continuing, Edwards explicitly discloses in col. 3, lines 3-15 that *in vitro* translated proNGF was aberrant in several respects. According to Edwards, "when NGF-beta mRNA obtained from mouse submaxillary gland was translated in cell-free expression systems, anti-NGF IgG failed to precipitate significant amounts. Some precipitate ensued when the mRNAs were translated cell-free in the presence of anti-NGF IgG, however, no biological activity was reported" (Edwards at col. 3, lines 4-9; emphases added). Additionally, Edwards further discloses that "expression of the pro-NGF-beta polypeptide in cell-free systems results in an improperly-folded protein, which is degraded rather than cleaved to active form by NGF-gamma" (Edwards at col. 3, lines 12-15; emphasis added).

Taken together, these statements expressly rebut the Patent Office's contention that *in vitro* translated proNGF, even if it were purified to at least 90% purity, would be predicted to have any biological activity at all, let alone a biological activity *in vivo* that is analogous to β-NGF as recited in claim 8.

And finally, appellants respectfully submit that the Patent Office's reliance on M.P.E.P. § 2123 is misplaced in the context of the instant rejection. According to the Patent Office, "[t]he use of patents as references is not limited to what the patentees describe as their own inventions or to the problems with which they are concerned. They are part of the literature of the art, relevant for all they contain", and that "[a] reference may be relied upon for all that it would have reasonably suggested to one

having ordinary skill the art, including nonpreferred embodiments". It appears that these quotations come from several cases decided by the CAFC: *In re Heck*, 699 F.2d 1331, 1332-33 (Fed. Cir. 1983); *Merck & Co. v. Biocraft Laboratories*, 874 F.2d 804 (Fed. Cir. 1989), *cert. denied*, 493 U.S. 975 (1989); *Upsher-Smith Labs. v. Pamlab, LLC*, 412 F.3d 1319, 1323 (Fed. Cir. 2005); and *Celeritas Technologies Ltd. v. Rockwell International Corp.*, 150 F.3d 1354, 1361 (Fed. Cir. 1998).

These cases fall into two broad categories: *Merck* and *Heck* are cases that involve the prior art effects of patents in the context of 35 U.S.C. § 103. Thus, they do not support the instant rejection, which is a rejection under 35 U.S.C. § 102.

Upsher-Smith and *Celeritas* relate to issues wherein the later claimed subject matter was explicitly disclosed in the prior art reference, but that the prior art reference taught away from the later claimed subject matter. Appellants respectfully submit that these cases also fail to support the instant rejection because Edwards does not explicitly disclose pharmaceutical preparations comprising purified human proNGF as the active ingredient, wherein the purified human proNGF is purified to at least 90% purity and has an activity in vivo analogous to β-NGF and promotes survival of dorsal root ganglia (DRG) sensory neurons as recited in claim 8. Therefore, appellants respectfully submit that even when viewed in the context of M.P.E.P. § 2123, Edwards fails to support the instant rejection.

Therefore, for this additional reason, appellants respectfully submit that Edwards does not disclose each and every element of independent claim 8, and thus does not support a rejection of independent claim 8 under 35 U.S.C. § 102(b). Appellants thus respectfully request that the instant rejection be reversed.

A.1.c. Edwards does not disclose any preparation wherein human proNGF is purified to at least 90% purity

Independent claim 8 also recites *inter alia* that the purified human proNGF is purified to at least 90% purity in the instantly claimed pharmaceutical preparations. Appellants respectfully submit that the Patent Office presented no evidence that human proNGF is present in any preparation disclosed in Edwards at a purity of at least 90%.

Initially and has set forth hereinabove, appellants respectfully submit that there are no compositions disclosed in Edwards that actually comprise human proNGF. The only compositions actually produced in Edwards are compositions of mouse proNGF. As such, appellants respectfully submit that there is no basis for the Patent Office to conclude that Edwards discloses human proNGF that has been purified to at least 90% purity.

Continuing, the Patent Office asserted in support of the instant rejection "the *in vitro* translated pro-NGF-beta solution taught in Example 2 of Edwards 'would therefore reasonably be purified to at least 90% purity based upon this translation system" (*see* Final Official Action at pages 2-3, *citing* the Final Official Action dated October 8, 2008 at page 5). It is reiterated that the "*in vitro* translated pro-NGF-beta solution taught in Example 2 of Edwards" is murine pro-NGF-beta. Thus, even assuming *arguendo* that it were "purified to at least 90% purity" (which for the reasons set forth herein, appellants respectfully submit it is likely not), appellants respectfully submit that the Patent Office's speculation with respect to this issue fails to support the instant rejection.

Additionally, appellants respectfully submit that the Patent Office appears to be relying on an inherency theory to support the assertion that *in vitro* translation produces

proteins that are at least 90% pure. For example, the Patent Office asserted that "because no side-by-side comparison has been provided by Applicants to alternatively demonstrate that the *in vitro* translation system cannot produce products that are at least 90% pure... Applicants' arguments remain not persuasive" (*see* Final Official Action at page 3).

Initially, appellants respectfully submit that the Patent Office's attempt to require a showing that *in vitro* translation systems *cannot* produce products that are at least 90% pure represents an improper attempt to shift the burden to appellants to prove a negative. The present issue with respect to asserted inherent anticipation of the subject matter of claim 8 is not whether an *in vitro* translation reaction *could possibly* produce an *in vitro* translated product of at least 90% purity. Rather, the issue is whether all *in vitro* translation reactions necessarily produce products of at least 90% purity. Appellants respectfully submit that one of ordinary skill in the art would understand that this is not the case. As such, it is believed that the Patent Office's mere speculation with respect to this issue is insufficient to switch the burden to appellants, particularly in light of the fact that Edwards does not present any data that would appear to suggest that the *in vitro* translated products are in fact purified to the requisite extent.

Additionally, appellants respectfully submit that in order to shift the burden to appellants, the Patent Office must at least demonstrate that the prior art discloses a composition that "appears to be 'either identical with or only slightly different than' a claimed composition" (*see Ex parte Gray*, 10 USPQ2d at 1925). In the context of the instant rejection, appellants respectfully submit that the showing required by the Patent Office to shift the burden is not that the product of the *in vitro* translation is asserted to

be pro-NGF-beta, but rather would be that the product of the *in vitro* translation appears to be at least about 90% pure. The Patent Office must provide evidence that the element at issue (*i.e.*, at least 90% purity) appears to be inherently disclosed. Edwards provides no such evidence, and thus appellants respectfully reiterate that the Patent Office's mere speculation with respect to this issue fails to switch the burden to appellants to prove otherwise.

Furthermore, and as described in more detail hereinabove, there are a considerable number of other biomolecules in an *in vitro* translation reaction, and there is no basis for the Patent Office to conclude that the translated product accounts for at least 90% of the mass of the product after *in vitro* translation.

Nonetheless, even assuming *arguendo* that an *in vitro* translation reaction could produce a product that is at least 90% pure, Appellants respectfully submit that the mere possibility that *in vitro* translation *might* produce sufficient translation product to be considered 90% pure is not sufficient to satisfy the requirements of inherent disclosure as set forth in *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) and M.P.E.P. § 2163.07(a).

Thus, the proper inquiry with respect to the Patent Office's reliance on inherency is whether *in vitro* translation of the murine proNGF coding sequences disclosed in Edwards would necessarily result in the *in vitro* translated murine proNGF polypeptide being 90% pure in the translation reaction. Appellants respectfully submit that the Patent Office provides no reasonable basis for concluding that it would given that it is known that *in vitro* translation systems include the translation machinery, which itself includes numerous proteins.

Appellants further respectfully submit that it is also known in the field of molecular biology that in order to visualize *in vitro* translated products, one or more radiolabeled amino acids are typically included in the reaction mix. This is at least partially due to the fact that unlabeled *in vitro* translated products typically cannot be distinguished from the proteins present in the translation systems itself when the reaction products are separated on standard polyacrylamide gels. The clear implication of this general knowledge is that *in vitro* translation products rarely, if ever, account for 90% of the mass of a completed *in vitro* translation reaction.

Summarily, appellants respectfully submit that the Patent Office presented no evidence sufficient to support its contention that any *in vitro* translation product is present in at least 90% purity in any *in vitro* translation reaction. Given this uncertainty, appellants respectfully submit that the Patent Office cannot properly rely on an inherency theory to support the instant basis for the rejection of claim 8 under 35 U.S.C. § 102(b).

Therefore, for this additional reason, appellants respectfully submit that Edwards does not disclose each and every element of independent claim 8, and thus does not support a rejection of independent claim 8 under 35 U.S.C. § 102(b). Appellants thus respectfully request that the instant rejection be reversed.

A.2. Argument for dependent claims 20, 26, 27, and 28

Appellants respectfully submit that claims 20, 26, 27, and 28 all depend directly or indirectly from claim 8. Thus, it is believed that these claims have been distinguished over Edwards for at least the reasons set forth hereinabove with respect to claim 8.

Additionally, appellants respectfully submit that Edwards does not disclose any pharmaceutical preparations wherein the purified human proNGF is a recombinant human proNGF, and thus claim 20 is believed to be distinguished over Edwards for this additional reason.

With respect to claim 26, appellants respectfully submit that this claim recites *inter alia* that the human proNGF present in the pharmaceutical preparation has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis. Given that Edwards does not teach any assays of human proNGF, appellants respectfully submit that there is no basis for the Patent Office to conclude that Edwards anticipates dependent claim 26.

Furthermore, since Edwards explicitly discloses that their murine pro-NGF-beta preparations had "little or no activity in the DRG assay" (*see* Example 6 of Edwards at col. 9, lines 6-9), appellants respectfully submit that there is additionally no basis for the Patent Office to assert that the instantly claimed human proNGF preparations would necessarily have the level of activity recited in claim 26.

Turning now to dependent claims 27 and 28, the Patent Office asserts that the recombinant pro-NGF-beta solution can be "derived from humans (e.g., col. 4, lines 40-42 [of Edwards]), which inherently comprises SEQ ID NO: 4 and inherently is encoded by a nucleic acid comprising SEQ ID NO: 3" (*see* Final Official Action at pages 3-4). Appellants respectfully submit that these assertions also amount to no more than speculation by the Patent Office, and thus do not satisfy the requirements for inherency set forth by the CAFC in *In re Robertson* (*see also* M.P.E.P. § 2163.07(a)).

Thus, it is believed that claims 20, 26, 27, and 28 are distinguished over Edwards at least based on their dependency from claim 8, as well as for the additional reasons set forth hereinabove. Accordingly, appellants respectfully request that the rejections of claims 20 and 26-28 under 35 U.S.C. § 102(b) over Edwards be reversed.

A.3. Argument for independent claim 29

Independent claim 29 has also been rejected under 35 U.S.C. § 102(b) upon the contention that Edwards anticipates this claim. According to the Patent Office, "[i]n that proNGF produced by [the procedures outlined in Edwards] inherently has whatever activity it possess based on its structural characteristics from which functional activity is directly derived, the limitations of claim[s 26 and] 29 are also reasonably met" (*see* Final Official Action at page 4).

Appellants respectfully submit that the arguments set forth hereinabove with respect to the rejection of claim 8 are also believed to be applicable to the instant rejection. Namely, appellants respectfully submit that Edwards does not teach any pharmaceutical compositions of human proNGF at all, and thus it is improper for the Patent Office to construe Edwards to teach a pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the purified human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis as recited in claim 29.

Furthermore, it is noted that since Edwards does not teach any pharmaceutical preparations of human proNGF, the Patent Office's premise that "proNGF produced by the procedures outlined in Edwards inherently have whatever activity it possesses" is

not sufficient *per se* for Edwards to support a rejection of claim 29 under 35 U.S.C. § 102(b). Appellants respectfully submit that in order to anticipate claim 29, Edwards would have to disclose pharmaceutical preparations of human proNGF that necessarily have a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis. As set forth hereinabove, Edwards does not teach any pharmaceutical compositions of human proNGF at all, and thus a condition precedent upon which *Ex parte Gray* and *In re Best* relied on by the Patent Office was based is entirely missing from the current facts.

To elaborate, in *Ex parte Gray*, the Board held that when a prior art composition appears to be "either identical with or only slightly different than" a claimed composition, the burden of persuasion can switch to appellants to demonstrate unexpected properties of their claimed composition (*see Ex parte Gray*, 10 USPQ2d at 1925). In order to switch the burden, however, the prior art composition must be identical with or only slightly different from appellants claimed composition. Stated another way, the holding of *Ex parte Gray* would be applicable to the instant analysis if and only if Edwards in fact disclosed a pharmaceutical composition comprising human proNGF as recited in instant claim 29. As appellants have demonstrated hereinabove, Edwards discloses no such composition, and thus the holding of *Ex parte Gray* is believed to be wholly inapplicable to the instant examination.

Furthermore, even if *Ex parte Gray* were to apply, appellants respectfully submit that the Patent Office has not satisfied its burden in switching the burden of persuasion to appellants because the record taken as a whole already provides ample evidence of the unexpected properties of the presently claimed pharmaceutical compositions.

Particularly, appellants respectfully submit that Edwards itself explicitly discloses that pro-NGF-beta has "little or no activity" in the DRG assay. The instant application, on the other hand, clearly discloses that the human proNGF of the instantly claimed pharmaceutical compositions promote survival of DRG sensory neurons, and that the activity of the human proNGF is about half that of human β-NGF in the same assay on a molar basis. As such, the record when taken as a whole already provides the evidence that the Patent Office might seek under *Ex parte Gray*, which appellants respectfully submit appears to have been ignored by the Patent Office to date.

Turning now to *In re Best*, appellants respectfully submit that the arguments presented hereinabove are equally applicable to distinguish this decision by the predecessor court of the CAFC. Particularly, appellants respectfully submit that unlike the situation in *In re Best*, the asserted prior art does not teach a composition that is "identical or substantially identical" to the instantly claimed pharmaceutical compositions. Additionally, even if Edwards did disclose such a composition, appellants respectfully submit that the record already demonstrates that the claimed pharmaceutical compositions have unexpected properties relative to any composition allegedly disclosed in Edwards, and thus appellants' burden of persuasion is already met.

Summarily, appellants respectfully submit that the Patent Office has not presented a *prima facie* case of anticipation of claim 29 over Edwards. As a result, appellants respectfully request that the instant rejection of claim 29 under 35 U.S.C. § 102(b) should be reversed.

B. Rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 103(a) as being unpatentable over Gray and Collins

B.1. Argument for independent claim 8

Claim 8 has been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Gray and Collins. Appellants respectfully submit that the combination of Gray and Collins does not support a *prima facie* case of obviousness of claim 8.

In support of the instant rejection of claim 8, the Patent Office asserted that Gray teaches both the amino acid and nucleotide sequence of human proNGF, methods of making NGF proteins recombinantly using either prokaryotic or eukaryotic host cells, and pharmaceutical compositions thereof. The Patent Office conceded, however, that Gray is silent regarding the activity of proNGF as it relates to β-NGF. This deficiency was asserted to be cured by Collins, which the Patent Office contended teaches "production of purified forms of all members of the NGF/BDNF family of neurotrophic proteins which would be valuable as pharmaceutical preparations", as well as biologically active recombinant human NGF family member proteins" (see Non-Final Official Action dated February 19, 2008 at page 7, which has been incorporated by reference by the Patent Office in the Final Official Action at page 5).

Appellants respectfully submit that the above assertions fail to support the instant rejection of claim 8. Particularly, appellants respectfully submit that when taken as a whole as required under M.P.E.P. § 2141.03, it is clear that one of ordinary skill in the art would not have been motivated to produce a pharmaceutical preparation comprising human proNGF as the active ingredient, wherein the purified human proNGF is purified

to at least 90% purity and has an activity in vivo analogous to β-NGF and promotes survival of dorsal root ganglia (DRG) sensory neurons as recited in claim 8.

To elaborate, appellants have argued during the instant prosecution that the Patent Office has not considered the prior art as a whole in the instantly presented rejection. Particularly, appellants have argued that there is no teaching or suggestion in the combination of Gray and Collins that a human proNGF would have activity in vivo analogous to β-NGF, and thus there is nothing in this combination that would suffice to overcome the express disclosure in Edwards that proNGF has "little or no" biological activity. As such, appellants have argued that the prior art taken as a whole provides neither suggestion nor motivation to produce the pharmaceutical composition of claim 8, nor does it provide any reasonable expectation that a pharmaceutical composition of human proNGF would have the activity recited in claim 8. Thus, appellants respectfully submit that the combination of Gray and Collins fails to support the instant rejection.

In response to these arguments as previously presented by appellants, the Patent Office stated:

> Applicants argue on pages 6-9 of the response that "applicants respectively traverse the Patent Office's assertion that the teachings of Edwards are not relevant to the instant rejection", based on the incorrect assumption that "Edwards' specific disclosure that pro-NGF is biologically inactive per se". In contrast to Applicants' unfortunate misrepresentation of the record, this is a rejection over Gray & Ullrich (U.S. Patent 5,169,762) and Collins et al (U.S. Patent 5,235,043); not a rejection over Edwards, wherein attempts to argue that any prior art's pro-NGF preparations are "biologically inactive per se" simply cannot be supported by taking a passage out of context from a single reference (i.e., Edwards), which is further not part of the pending rejection.

Final Official Action at page 5. Appellants respectfully submit, however, that it is the Patent Office that has misconstrued the implication of Edwards express teachings with

respect to the instant rejection. Given that Edwards specifically teaches that proNGF itself has little or no biological activity, the issue with respect to how Edwards impacts the instant rejection is that when taken as a whole the prior art as of the filing date of the instant reference taught that proNGF is biologically inactive *per se*. The Patent Office has failed to consider that the appropriate time frame for analyzing the prior art is as of the filing date of the instant application, and in doing so, the prior art must be considered as a whole.

Therefore, appellants respectfully submit that contrary to the Patent Office's apparently misguided assertion, it is not an "unfortunate misrepresentation of the record" to point out how Edwards impacts the instant rejection. Whether or not, as the Patent Office has argued, the instant rejection is a rejection over Gray and Collins and not a rejection over Edwards, the express teachings of Edwards cannot be disregarded. Appellants respectfully submit that it is clear error for the Patent Office to ignore Edwards by attempting to limit the scope of the analysis with respect to the instant rejection to consideration of only the disclosures of Gray and Collins.

Accordingly, and in contrast to the Patent Office's assertion, appellants respectfully submit that their arguments are based on the totality of the evidence (*i.e.*, the combination of Gray and Collins also in light of Edwards), and are not, as the Patent Office has incorrectly asserts, based upon "taking a passage out of context from a single reference (i.e., Edwards), which is further not part of the pending rejection" (*see* Final Official Action at page 5).

Furthermore, appellants respectfully submit that when examining claims under 35 U.S.C. §103(a), the Patent Office must consider what the prior art taken as a whole

reasonably suggests to one of ordinary skill in the art. With respect to the instant rejection, the Patent Office appears to have asserted that appellants have *incorrectly assumed* that Edwards disclosed that pro-NGF is biologically inactive per se (*see* Final Official Action at page 5). Appellants respectfully submit, however, that the only disclosure in Edwards with respect to any biological activities of pro-NGF-β can be found in Example 6 of Edwards as follows:

> The results indicate that supernatant from L929/VV:wt exhibited no NGF activity, supernatant from L929/VV:NGF-A and L929/VV:NGF-B (not digested) exhibited little to no activity, and supernatant from L929/VV:NGF-A and L929/VV:NGF-B digested with trypsin exhibited substantial NGF-beta activity.

Edwards at col. 9, lines 6-11. Review of Edwards indicates that "VV:wt" was a negative control wild type viral vector that does not encode any NGF polypeptide, and VV:NGF-A and W:NGF-B were vectors that encoded different forms of murine pro-NGF-beta. Since VV:wt did not encode any NGF polypeptide, it would not have been expected to exhibit any NGF activity. The supernatants of cells infected with VV:NGF-A and VV:NGF-B (not digested) were also reported to have "little or no activity", whereas "L929/VV:NGF-A and L929/VV:NGF-B digested with trypsin exhibited substantial NGF-beta activity". Appellants respectfully submit that the clear implication of these passages is that until pro-NGF-beta is digested with trypsin (which Edwards discloses activates pro-NGF-beta in a manner similar to the natural activator NGF-gamma; *see e.g.*, Edwards at col. 4, lines 25-38) or an equivalent protease, it has little or no biological activity. Given that one of ordinary skill in the art would also understand that many biologically active polypeptides are synthesized *in vivo* in an inactive "pro" form, one of ordinary skill in the art would also find the results of the experiments disclosed in

Edwards to be completely consistent with the expected function of pro-NGF-beta. Therefore, contrary the Patent Office's attempt to portray appellants' characterization of Edwards as "an incorrect assumption", appellants respectfully submit that when taken as a whole, the clear suggestion in Edwards is that pro-NGF-beta indeed has little or no biological activity *per se*.

Next, in addressing appellants' arguments, the Patent Office asserted that although Collins is silent regarding the activity of proNGF as it relates to β-NGF, the activity of proNGF is directly related to its structure, and therefore, is an inherent property of proNGF. According to the Patent Office,

> the issue remains that MPEP 2112 makes clear that "The express, implicit, and inherent disclosures of a prior art reference may be relied upon in the rejection of claims under 35 U.S.C. 102 or 103. "The inherent teaching of a prior art reference, a question of fact, arises both in the context of anticipation and obviousness." In re Napier, 55 F.3d 610,613,34 USPQ2d 1782, 1784 (Fed. Cir. 1995) (affirmed a 35 U.S.C. 103 rejection based in part on inherent disclosure in one of the references). See also In re Grasselli, 713 F.2d 731,739,218 USPQ 769, 775 (Fed. Cir. 1983)."

Final Official Action at page 5. Appellants respectfully submit, however, that both the claimed subject matter and the prior art also must be considered as a whole, which includes consideration of all disclosures that teach away from the claimed invention (M.P.E.P. § 2141.03).

To elaborate, appellants respectfully submit that as of the filing date of the instant application, one of ordinary skill in the art would have believed that proNGF *per se* had little or no activity based on the express disclosure of Edwards. The Patent Office has presented no evidence that rebuts this clear suggestion in Edwards. Appellants further respectfully submit that the instant claims are directed to pharmaceutical compositions comprising human proNGF as the active ingredient. As such, it is believed to be clear

that Edwards teaches away from pharmaceutical compositions comprising human proNGF as the active ingredient because "active ingredients" would not have "little or no activity" in pharmaceutical compositions.

Furthermore, as set forth in more detail hereinabove, inherency "may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient"; *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999); emphases added). *See also* M.P.E.P. § 2163.07(a). Edwards expressly stated that the pro-NGF-beta preparations disclosed therein had little or no activity. Thus, appellants respectfully submit that as of the filing date of the instant application, it was believed that proNGF had no inherent biological activity. Thus, the fact that proNGF was later found to have biological activity *per se* cannot support the instant rejection because it is only with impermissible hindsight that the Patent Office can assert that the combination of Gray and Collins would suggest the subject matter of claim 8.

Continuing, the Patent Office also argued that *KSR International Co. v. Teleflex Inc.*, 550 U.S. 398 (2007) stands for the proposition that "the simple substitution of one known, equivalent element [i.e., proNGF for NGF] for another to obtain predictable results [i.e., increase DRG neuronal survival], or the combining of prior art elements [i.e., Collins' proneurotrophin polypeptides for the proNGF polypeptide] according to known methods [of making recombinant polypeptides] to yield predictable results [i.e., increase survival of DRG neurons], reasonably supports a *prima facie* case of obviousness" (*see* Final Official Action at page 8). Thus, according to the Patent Office,

proNGF and NGF are "known equivalents", and increasing DRG neuronal survival is a "predictable result". Appellants respectfully traverse both of these assertions.

First, appellants respectfully submit that *KSR* held that "the simple substitution of one known, equivalent element for another to obtain predictable results" *might* be obvious. However, it is implicit in this holding that the elements must be known to be equivalents, and the results must be predictable. Particularly, appellants respectfully submit that as of the filing date of the instant application, the prior art clearly provided no basis for concluding that proNGF and NGF were biological equivalents, and further provided no basis to conclude that one of ordinary skill in the art could have predictably produced a pharmaceutical preparation of proNGF as the active ingredient.

Turning first to the asserted equivalency of proNGF and NGF, appellants have argued that these two polypeptides would not have been properly considered equivalents as of the filing date of the instant application. Particularly, appellants have argued that since there was no disclosure prior to appellants' specification that proNGF had any biological activities, one of ordinary skill in the art would not have believed that proNGF and NGF were equivalents in a DRG assay as was asserted by the Patent Office. The Patent Office argued however, that appellants' argument that proNGF is not an equivalent of NGF "simply is not correct, because proneurotrophic factors are well known to be the precursors of neurotrophic factors, such as proNGF is the precursor of NGF-beta" (Final Official Action at page 6) or because "it is the Examiner's position that prodrugs that are metabolized into drugs are 'equivalants' [sic], and add no additional contribution to the art; absent evidence to the contrary, which Applicants have only attempted to support by a single passage taken out of context from a single reference

(i.e., Edwards), while ignoring the other teachings made of record" (Final Official Action at page 7).

Appellants respectfully disagree with both of the Patent Office's contentions. Appellants respectfully submit that the Patent Office's line of argument misses the point. The proper inquiry is not whether proNGF *could* give rise to NGF-beta under some unknown circumstance. Rather, appellants respectfully submit that the proper inquiry with respect to the instant rejection is whether as of the filing date of the instant application, one of ordinary skill in the art would have understood proNGF and β-NGF to be interchangeable with respect to the relevant function (*i.e.*, promoting survival of DRG sensory neurons). Given that the only disclosure that provides any reasonable expectation that proNGF could function in a DRG assay is present within appellants' own specification, there is no basis upon which the Patent Office could conclude that one of ordinary skill in the art would have recognized proNGF and β-NGF to be equivalents as of the filing date of the instant application. Thus, appellants respectfully submit that the Patent Office's attempted "equivalency" basis also fails to support the instant rejection.

Continuing, in support of the equivalency of proNGF and β-NGF, the Patent Office also asserted that Collins teaches "that it was well accepted in the art that 'the proper folding and assumption of biological activity of mature NGF will only occur if it is first synthesized as the full-length precursor, as occurs in eukaryotic cells and in natural sources' (i.e., col. 32, lines 62-65); thereby, providing motivation for making human proNGF protein nonetheless" (Non-Final Official Action dated February 19, 2008 at page 7). Appellants respectfully submit that this assertion also fails to support the

instant rejection, and in fact is explicitly contradicted by Edwards, which is believed to be representative of the understanding in the art as a whole as of the filing date of the present U.S. patent application.

To elaborate, appellants respectfully submit that review of Examples 3 and 4 of Edwards show that W:NGF-A (Example 4) is a viral vector encoding a full-length murine NGF polypeptide, which was expressed in mouse L929 cells. The full-length NGF polypeptide included the pro-NGF-beta sequences, and thus the "full-length precursor" was synthesized. Mouse L929 cells are eukaryotic cells, and thus, consistent with Collins, Edwards taught synthesizing mature NGF as a full-length precursor in eukaryotic cells, and yet the pro-NGF-beta polypeptide so produced itself had little or no biological activity. As such, appellants respectfully submit that this assertion, even if true, fails to support the instant rejection.

Continuing, the Patent Office also asserts that appellants have argued the Gray and Collins references individually. Appellants respectfully disagree. Appellants respectfully submit that the Patent Office's assertion that Gray teaches both the amino acid and nucleotide sequence of human proNGF, methods of making NGF proteins recombinantly using either prokaryotic or eukaryotic host cells, and pharmaceutical compositions thereof when combined with Collins' asserted teaching of "production of purified forms of all members of the NGF/BDNF family of neurotrophic proteins which would be valuable as pharmaceutical preparations", as well as biologically active recombinant human NGF family member proteins" cannot be read to disclose or suggest a pharmaceutical preparation of human proNGF as the active ingredient as recited in claim 8, particularly in view of the clear suggestion in Edwards that pro-NGF-

beta had no biological activity *per se* that remains uncontroverted by any evidence on the record. Thus, it is appellants' contention that as of the filing date of the instant application, one of ordinary skill in the art would not have been motivated to produce the subject matter of instant claim 8 because it would have been believed that human proNGF could not function as an active ingredient based on Edwards. Thus, appellants respectfully submit that they are not arguing the references individually, but instead are considering the prior art as a whole, including the cited combination, as is required by M.P.E.P. § 2141.03.

Next, the Patent Office asserts that "the fact that applicant has recognized another advantage which would flow naturally from following the suggestion of the prior art cannot be the basis for patentability when the differences would otherwise be obvious" (emphasis added). See *Ex parte Obiaya*, 227 USPQ 58, 60 (Bd. Pat. App. & Inter. 1985). Appellants respectfully submit, however, that the Patent Office provides no basis for concluding that the "advantage discovered" by appellants would "flow naturally from following the suggestion of the prior art". As set forth herein in more detail, it is appellants' position that there is no suggestion in the prior art to produce the subject matter of claim 8. As such, what would "flow naturally" from the prior art when taken as a whole would be the understanding that human proNGF would have no biological activity *per se*, and thus the prior art taken as a whole teaches against the subject matter of claim 8. Thus, appellants respectfully submit that the Patent Office's reliance on *Ex parte Obiaya* is believed to be flawed.

Continuing, the Patent Office also asserts that Collins suggests employing human proNGF in a pharmaceutical composition. Appellants respectfully disagree.

Review of Collins demonstrates that contrary to the Patent Office's assertion, the only pharmaceutical compositions disclosed in Collins relate to mature forms of biologically active NGF. This is shown in, for example, col. 3, lines 10-14 ("Recombinant expression systems that are capable of producing the large quantities of fully-biologically active and structurally-unmodified mature NGF needed for pharmaceutical development and for the treatment of patients"); in col. 4, lines 23-24 ("mature human NGF has been unavailable in sufficient amounts for pharmaceutical use"); and in col. 4, lines 45-51 ("a manufacturing system capable of producing fully-biologically-active and chemically-unmodified human mature NGF in large amounts in bacteria will be useful in producing similar large amounts of any member of the NGF/BDNF family in a biologically-active and unmodified form suitable for pharmaceutical use") of Collins (emphases added). As a result, appellants respectfully submit that there is believed to be no disclosure in Collins that unambiguously describes or suggests pharmaceutical compositions of proNGF or any other proneurotrophic factors.

Additionally, the Patent Office asserted that it would have been obvious to make and purify human proNGF to homogeneity based on the teachings of both Gray and/or Collins using standard purification techniques known in the art, or as described by both Gray or by Collins, etc. either for use in pharmaceutical compositions, as suggested by Collins, in which the subsequent purification would reasonably minimize undesirable side effects and/or adverse immunological concerns well known in the art (thereby, increasing the number of neurotrophic proteins valuable for treating neurodegenerative diseases, as allegedly suggested by Collins, or for use of human proNGF as a prodrug. Appellants respectfully disagree. Whether or not human proNGF could have been

produced by standard techniques is not the issue. Again, claim 8 recites a pharmaceutical composition comprising human proNGF as the active ingredient. The mere fact that one of ordinary skill in the art might have been capable of purifying human proNGF does not imply that one of ordinary skill in the art would have been motivated to produce a pharmaceutical composition wherein the human proNGF was the active ingredient. Appellants respectfully submit that this assertion also fails to support the instant rejection of claim 8 because it fails to consider both claim 8 and the prior art in their entireties.

The Patent Office also asserted that the combination of Gray and Collins renders obvious the use of human proNGF as a prodrug for its eventual processing into a biologically active and mature NGF form. Appellants respectfully submit, however, that a pharmaceutical composition comprising human proNGF as a prodrug would not be a considered by one of ordinary skill in the art to be a pharmaceutical preparation with purified human proNGF as the active ingredient since the term "prodrug" itself indicates that the agent is inactive. As such, appellants respectfully submit that this contention fails to consider claim 8 as a whole, and thus fails to support the instant rejection.

Next, the Patent Office asserted that no product-by-process steps are recited in the current claims (*See* Final Official Action at page 9). Appellants respectfully submit that the Patent Office has not provided any explanation as to how this assertion might support the instant rejection. Appellants respectfully submit that the subject matter of instant claim 8 *per se* is believed to be distinguished over the combination of Gray and Collins.

And finally, even if the Patent Office were to have established a *prima facie* case of obviousness of claim 8 over Gray and Collins, appellants have rebutted the *prima facie* case with evidence of unexpected results. Specifically, appellants respectfully submit that the evidence of record provides ample support for the contention that it would have been unexpected that proNGF *per se* would have biological activity.

To elaborate, appellants respectfully reiterate that Edwards explicitly discloses that proNGF had "little or no activity" in a standard DRG assay. The instant application, on the other hand, clearly discloses that the activity of proNGF in this exact same assay is about half that of human β-NGF in the same assay on a molar basis, which appellants respectfully reiterate would not have been interpreted by one of ordinary skill in the art as "little or no activity". As such, appellants respectfully submit that the instant application *per se* provides evidence that proNGF has biological activity that is completely contrary to what was believed to have been known as of the filing date of the instant application, and thus represents an unexpected result that rebuts the Patent Office's asserted *prima facie* case (*see* M.P.E.P. § 2141.01).

Additionally, appellants have submitted further evidence of the unexpected properties of proNGF. In various experiments described in a Declaration by Dr. Susan Lorey under 37 C.F.R. 1.132 received by the Patent Office on January 2, 2006 (hereinafter the "2006 Lorey Declaration"; a true and accurate copy of which is being submitted herewith in the Evidence Appendix, Section IX herein below), it was determined that "proNGF had significantly greater efficacy than β-NGF in inducing murine fibroblast migration" (*see* 2006 Lorey Declaration, pages 1-2, Point 2). The 2006 Lorey Declaration also described experiments that demonstrated that at the same

concentration, "proNGF... had significantly greater efficacy that β-NGF... in inducing bovine epithelia cell migration" (*see* 2006 Lorey Declaration, page 2, Point 3). Taken together, these experiments led Dr. Lorey to conclude:

> proNGF itself acts as an active ingredient. In support of this conclusion, it is noted that if proNGF exhibited its activity only after cellular cleavage of the proNGF into β-NGF, proNGF could not have greater activity than β-NGF as observed in these experiments.

2006 Lorey Declaration at page 2 (emphasis added).

Other researchers also reported finding that proNGF *per se* has biological activity after the filing of the instant U.S. patent application. These include the experiments reported in the Fahnestock *et al.* Abstract submitted to the Patent Office and received on January 2, 2006 (corresponds to Fahnestock *et al.* (2004) *J Neurochem* 89:581-592; a true and accurate copy of which is being submitted herewith in the Evidence Appendix, Section IX herein below), which disclosed that proNGF exhibits neurotrophic activity similar to mature 2.5S NGF".

Also, Nykjaer *et al.* reported in 2004 that sortilin is a receptor that binds to proNGF but not to mature β-NGF, to "selectively induce p75NTR-dependent apoptosis in neurons, smooth muscle cells, and oligodendrocytes" (Nykjaer *et al.* (2004) *Nature* 427:843-848 (*see* pages 843-844, bridging paragraph); submitted to the Patent Office and received on January 2, 2006; a true and accurate copy of which is being submitted herewith in the Evidence Appendix, Section IX herein below). Nykjaer *et al.* also discloses that "proNGF targets and promotes formation of a signaling complex comprising endogenous sortilin and $p75^{NTR}$, and that both receptors are required for pro-NGF mediated apoptosis (*see* Nykjaer *et al.* at page 847 (left column, first full paragraph).

As such, appellants respectfully submit that as of the filing date of the instant application, one of ordinary skill in the art would not have believed that proNGF had biological activity *per se*, and thus the discovery by appellants that it did in fact have its own biological activities represents an unexpected result that rebuts the Patent Office's asserted *prima facie* case of obviousness over the combination of Gray and Collins.

Accordingly, appellants respectfully submit that the instant rejection of claim 8 is based on a failure by the Patent Office to consider both the prior art and the claims as wholes, and further requires hindsight based on knowledge found only in appellants' specification. Taken together, these errors compel the conclusion that the rejection of claim 8 under 35 U.S.C. § 103(a) over the combination of Gray and Collins must be reversed.

B.2. Argument for dependent claims 20, 26, 27, and 28

Appellants respectfully submit that claims 20, 26, 27, and 28 all depend directly or indirectly from claim 8. Thus, it is believed that these claims have been distinguished over the combination of Gray and Collins for at least the reasons set forth hereinabove with respect to claim 8.

With respect to claim 26, appellants further respectfully submit that this claim recites *inter alia* that the human proNGF present in the pharmaceutical preparation has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis. Appellants respectfully submit that given the express disclosure of Edwards, again as a representative of the understanding of one of ordinary skill in the art, there is no basis upon which the Patent Office could reasonably conclude that it would be predictable that the human proNGF in

the pharmaceutical composition of claim 26 would be about half as active as mature human β-NGF is a DRG assay. Therefore, appellants respectfully submit that the instant rejection as applied to claim 26 is inconsistent by the Supreme Court's holding in *KSR* that modifications of the prior are must yield predictable results to support a rejection under 35 U.S.C. §103(a).

Turning now to dependent claims 27 and 28, the Patent Office asserts that "Gray et al teach both the amino acid and nucleotide sequence of human proNGF" (*see* Final Official Action at page 8; *citing* Figures 4-6). Appellants respectfully submit, however, that the Patent Office has not indicated precisely where Gray discloses SEQ ID NO: 3 or SEQ ID NO: 4.

Appellants further respectfully submit that review of the sequences presented in Figures 4-6 of Gray indicates that there are no sequences disclosed in this patent that comprise SEQ ID NO: 3 or SEQ ID NO: 4 as recited in claims 27 and 28. For example, SEQ ID NO: 3 includes the following sequence of nucleotides at its beginning (*i.e.*, at its 5' end): atg gaa cca cac tca gag agc aat gtc. Appellants respectfully submit that to the best of their knowledge, this sequence does not appear anywhere in Gray. As such, appellants respectfully submit that contrary to the Patent Office's assertion, Gray does not disclose either SEQ ID NO: 3 or SEQ ID NO: 4, and thus for this additional reason, claims 27 and 28 are believed to be distinguished over the combination of Gray and Collins.

Therefore, it is believed that claims 20, 26, 27, and 28 have been distinguished over the combination of Gray and Collins at least based on their dependency from claim 8, and also for the additional reasons set forth hereinabove. Accordingly, appellants

respectfully request that the rejections of claims 20 and 26-28 under 35 U.S.C. § 103(a) over the combination of Gray and Collins be reversed and that the claims be allowed at this time.

B.3. Argument for independent claim 29

Independent claim 29 has also been rejected under 35 U.S.C. § 103(a) upon the contention that the claim is obvious over the combination of Gray and Collins. It is noted, however, that the Patent Office presented no new arguments with respect to claim 29, merely relying on an inherency theory related to the activity of proNGF being directly related to its amino acid sequence.

Appellants respectfully submit that here as well, the arguments set forth hereinabove with respect to the rejection of claim 8 are also believed to be applicable to the instant rejection. Namely, appellants respectfully submit that the combination of Gray and Collins does not disclose or suggest ant pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the purified human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis as recited in claim 29.

Furthermore, appellants respectfully submit that the Patent Office's reliance on inherency is flawed as the Patent Office has not presented any arguments whatsoever to counter the express teaching away that is present in Edwards, which again is believed to be representative of the understanding of one of ordinary skill in the art. Particularly, appellants respectfully submit that under current case lase including the Supreme Court's holding in *KSR*, the Patent Office bears the burden of providing a reasoned argument as to why one of ordinary skill in the art would have believed as of

the filing date of the instant application that human proNGF could predictably serve as the active ingredient in a pharmaceutical composition. Appellants respectfully submit that the disclosure of proNGF as a biologically active agent *per se* can be found only in appellants' own specification, and thus cannot form the basis for a rejection of claim 29 under 35 U.S.C. §103(a).

Summarily, appellants respectfully submit that taken in combination with Edwards' teachings against proNGF as a pharmaceutically active agent, the Patent Office has not presented a *prima facie* case of obviousness of claim 29 over Gray and Collins. As a result, appellants respectfully request that the instant rejection of claim 29 under 35 U.S.C. § 103(s) should be reversed.

C. Rejection of claims 8, 20, and 26-29 under 35 U.S.C. § 103(a) as being unpatentable over Gray and/or Collins and/or JP 09-023883.

C.1. Argument for independent claim 8

Claim 8 has been rejected under 35 U.S.C. §103(a) upon the contention that the claim is obvious over Gray and/or Collins and/or JP 09-023883. In support of this rejection, the Patent Office reiterated its assertions with respect to the teachings of Gray and Collins. Additionally, the Patent Office asserted that JP 09-023883 taught improved methods for producing biologically active beta-NGF by solubilizing insoluble aggregates obtained from expression in *E. coli* and using a pulse regenerating treatment/denaturation/renaturation involving a redox system selected from cystamine/cysteamine and cysteine in the presence of arginine. The Patent Office

admits, however, that JP 09-023883 does not teach producing biologically active pro-NGF using this same improved methodology.

The Patent Office asserted, however, that it would have been obvious to one of ordinary skill in the art to make and purify human proNGF to homogeneity based on the teachings of both Gray and Collins using standard purification techniques known in the art, or as described by Gray or by Collins or as taught by JP 09-023883, either for use in pharmaceutical compositions, in which the subsequent purification would reasonably minimize undesirable side effects and/or adverse immunological concerns well known in the art (thereby, increasing the number of neurotrophic proteins valuable for treating neurodegenerative diseases, or for use of human proNGF itself as a prodrug for its eventual processing into a biologically active and mature NGF form, whose biological activity related to increasing DRG/sensory neuronal survival is well characterized within the art. The Patent Office further asserted that it would also have been obvious to one of ordinary skill in the art to make and purify human proNGF itself as a prodrug using JP 09-023883's improved method of denaturation/renaturation of NGF polypeptides, in order to maximize biological activity cost effectively, as taught by JP 09-023883, because proNGF is prodrug as it relates to NGF-beta, which would alternatively and naturally be processed into NGF-beta once administered to a patient, and/or have equivalent biological activity as NGF-beta, due to its overlapping amino acid sequence identity, with a reasonable expectation of success.

Appellants respectfully traverse the assertions upon which the Patent Office has attempted to support the instant rejection, and submit the following remarks.

Initially, appellants respectfully note that the Patent Office's attempt to argue that one of ordinary skill in the art would have been motivated to produce any biologically active proNGF is based on impermissible hindsight for the reasons set forth hereinabove. Appellants respectfully reiterate that before the disclosure of appellants' specification, it was believed that proNGF *per se* did not have any biological activity. Thus, appellants respectfully submit that there is no basis for the Patent Office to assert that it would have been obvious to employ any particular purification technique to produce the subject matter of claim 8.

Furthermore, appellants respectfully submit that under the holding of *KSR*, it would have to be predictable as of the filing date of the instant application that proNGF had the biological activities recited in instant claim 8. There is no evidence on the record that supports the contention that one of ordinary skill in the art would have believed that proNGF *per se* had biological activity analogous to β-NGF. Further, the teachings of Edwards that proNGF actually had little to no biological activity teaches against the subject matter of claim 8. Since the Patent Office must consider the prior art as a whole, the absence of any evidence from the record that contradicts the express teaching of Edwards is believed to be fatal to the Patent Office's current rejection of claim 8.

Additionally, appellants respectfully submit that given the teaching of Edwards, there would have been no basis for concluding that that the reason that the Edwards proNGF did not have any activity was because it was in the form of insoluble aggregates. It is noted that Edwards also disclosed preparations of murine proNGF that had been produced from eukaryotic cells. Since the Patent Office has already argued

that "the proper folding and assumption of biological activity of mature NGF will only occur if it is first synthesized as the full-length precursor (i.e., as a proneurotrophin, such as proNGF), as occurs in eukaryotic cells" and Edwards disclosed that proNGF-beta produced in mouse L929 cells had "little or no activity", appellants respectfully submit that there would have been no basis for one of ordinary skill in the art to conclude that a different purification scheme would have resulted in proNGF with any biological activity.

And finally, appellants respectfully submit that the evidence of record with respect to the biological activities of proNGF *per se* are sufficient to rebut the Patent Office's asserted *prima facie* case of obviousness of claim 8 over Gray and/or Collins and/or JP 09-023883.

Therefore, appellants respectfully submit that the Patent Office has not demonstrated that one of ordinary skill in the art would have been motivated to employ the production techniques of JP 09-023883 for any particular purpose, and that doing so would have been predicted to result in a pharmaceutical preparation with proNGF as the active agent as set forth in claim 8. As a result, appellants respectfully submit that claim 8 is nonobvious over Gray and/or Collins and/or JP 09-023883, and respectfully request that the instant rejection be reversed.

C.2. Argument for dependent claims 20, 26, 27, and 28

Appellants respectfully submit that claims 20, 26, 27, and 28 all depend directly or indirectly from claim 8. Thus, it is believed that these claims have been distinguished over Gray and/or Collins and/or JP 09-023883 for at least the reasons set forth hereinabove with respect to claim 8.

Additionally, with respect to claim 26, appellants respectfully reiterate that this claim recites *inter alia* that the human proNGF present in the pharmaceutical preparation has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis. Appellants respectfully submit that given the express disclosure of Edwards as representative of the understanding in the art, there is no basis upon which the Patent Office could reasonably conclude that it would be predictable that the human proNGF in the pharmaceutical composition of claim 26 would be about half as active as mature human β-NGF is a DRG assay. Therefore, appellants respectfully submit that JP 09-023883 fails to cure the deficiencies of Gray and Collins discussed hereinabove with respect to the previous rejection of claim 26 under 35 U.S.C. §103(a).

Turning now to dependent claims 27 and 28, the Patent Office asserts that "Gray et al teach both the amino acid and nucleotide sequence of human proNGF" (*see* Final Official Action at page 8; *citing* Figures 4-6). Appellants respectfully submit, however, that as set forth hereinabove, it does not appear that Gray discloses SEQ ID NO: 3 or SEQ ID NO: 4. Appellants further respectfully submit that JP 09-023883 fails to cure this deficiency. Thus, it is submitted that the combination of Gray and/or Collins and/or JP 09-023883 fails to support a rejection of claims 27 and 28 under 35 U.S.C. § 103(a).

Accordingly, it is believed that claims 20, 26, 27, and 28 have been distinguished over the combination of Gray and Collins and JP 09-023883 at least based on their dependency from claim 8, and also for the additional reasons set forth hereinabove. Accordingly, appellants respectfully request that the rejections of claims 20 and 26-28

under 35 U.S.C. § 103(a) over the combination of Gray and Collins and JP 09-023883 be reversed and that the claims be allowed at this time.

C.3. Argument for independent claim 29

Independent claim 29 has also been rejected under 35 U.S.C. § 103(a) upon the contention that the claim is obvious over the combination of Gray and Collins and JP 09-023883. It is noted, however, that the Patent Office again presented no new arguments with respect to claim 29 over those presented in support of the rejection of claim 29 over the previous combination of references.

With respect to this rejection, appellants respectfully submit that the arguments set forth hereinabove with respect to the rejection of claim 8 are also believed to be applicable to the instant rejection. Particularly, appellants respectfully submit that the combination of Gray and Collins and JP 09-023883 does not disclose or suggest any pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the purified human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis as recited in claim 29.

Appellants further respectfully reiterate that the Patent Office's reliance on inherency is flawed as it cannot reasonably be argued that it would have been predictable in light of Edwards that proNGF *per se* would have the recited biological activity as of the filing date of the instant application. Since the disclosure of proNGF as a biologically active agent *per se* can be found only in appellants' own specification, appellants respectfully submit that the instant rejection of claim 29 under 35 U.S.C. §103(a) employs impermissible hindsight bias.

Summarily, appellants respectfully submit that taken in combination with Edwards' teachings against proNGF as a pharmaceutically active agent, the Patent Office has not presented a *prima facie* case of obviousness of claim 29 over Gray and Collins and JP 09-023883. As a result, appellants respectfully request that the instant rejection of claim 29 under 35 U.S.C. § 103(a) should be reversed.

DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this paper to Deposit Account No. **50-0426**.

Respectfully submitted,

JENKINS, WILSON, TAYLOR & HUNT, P.A.

Date: 10/15/2010 By: 

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1406/415 AAT/CPP/dbp

VIII. Claims Appendix

8. A pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the purified human proNGF is purified to at least 90% purity and has an activity in vivo analogous to β-NGF and promotes survival of dorsal root ganglia (DRG) sensory neurons.

20. The pharmaceutical preparation of claim 8, wherein the purified human proNGF is recombinant human proNGF.

26. The pharmaceutical preparation according to claim 8, wherein the human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis.

27. The pharmaceutical preparation according to claim 8, wherein the purified human proNGF comprises an amino acid sequence as set forth in SEQ ID NO: 4.

28. The pharmaceutical preparation according to claim 8, wherein the purified human proNGF is encoded by a nucleic acid comprising SEQ ID NO: 3.

29. A pharmaceutical preparation comprising a pharmaceutically acceptable carrier and purified human proNGF as the active ingredient, wherein the purified human proNGF has a biological activity in a dorsal root ganglion (DRG) assay that is about half that of human β-NGF in the same assay on a molar basis.

IX. Evidence Appendix

IX.1. Declaration of Dr. Susan Lorey Pursuant to 37 C.F.R. 1.132

Applicant submitted a Declaration of Dr. Susan Lorey Pursuant to 37 C.F.R. 1.132 (referred to herein as the "2006 Lorey Declaration") that was received by the Patent Office on January 2, 2005. It is believed that the 2006 Lorey Declaration had been entered into the record as no objection to the 2006 Lorey Declaration was presented in the Non-Final Official Action dated April 3, 2006. A true and accurate copy of the 2006 Lorey Declaration is attached hereto.

IX.2. Fahnestock *et al* Abstract

Applicant submitted a true and accurate copy of the Abstract for Fahnestock *et al.* (2004) *J Neurochem* 89:581-592 that is available through the website of the National Center for Biotechnology Information (NCBI), which was received by the Patent Office on January 2, 2005. It is believed that the Fahnestock *et al.* Abstract had been entered into the record as no objection to it was presented in the Non-Final Official Action dated April 3, 2006. A true and accurate copy of the Fahnestock *et al.* Abstract is attached hereto.

IX.3. Nykjaer *et al.*

Applicant submitted a true and accurate copy of Nykjaer *et al.* (2004) *Nature* 427:843-848, which was received by the Patent Office on January 2, 2005. It is believed that the Nykjaer *et al.* journal article had been entered into the record as no objection to it was presented in the Non-Final Official Action dated April 3, 2006. A true and accurate copy of the Nykjaer *et al.* journal article is attached hereto.

Attorney's Docket No.: 13028-002001 / P12999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Anke Rattenholl et al. Art Unit : 1647
Serial No. : 09/807,096 Examiner : Robert C. Hayes
Filed : November 19, 2001
Title : METHOD FOR OBTAINING ACTIVE BETA-NGF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION BY DR. SUSAN LOREY UNDER 37 C.F.R. 1.132

I, **Susan Lorey**, hereby declare that:

1. I conducted or supervised the following two experiments, in which β-NGF and proNGF were compared for their ability to induce migration of (1) murine fibroblasts, and (2) bovine corneal endothelial cells.

In organisms cellular migration is of importance in all processes where cells have to be recruited to places they are required, e.g. migration of inflammatory cells, fibroblasts, endothelial or epithelial cells to support wound healing. *In vitro* cellular migration may be studied in the Boyden Chamber as model for examining cell movement through a porous membrane towards a chemoattractant.

The proNGF and β-NGF were generated as described in the Specification of US 09/807,096, e.g., at page 13, line 14 through page 25, line 26.

2. In a first experiment, embryonic murine fibroblasts (3T3) were used to study the effect of proNGF and β-NGF on the migration of these cells. Cellular migration of the cells stimulated

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

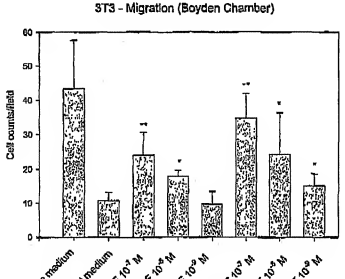
Date of Deposit

Signature

Typed or Printed Name of Person Signing Certificate

by proNGF, β-NGF or a test medium negative control was determined in a Boyden Chamber after a period of 4 hours.

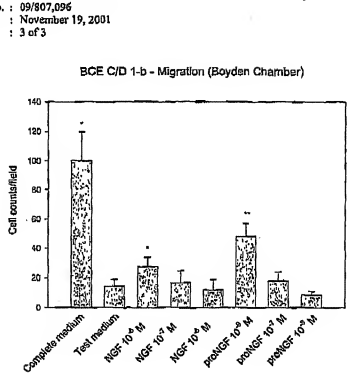
At all doses, proNGF had significantly greater efficacy than β-NGF in inducing murine fibroblast migration, as compared to the negative control.



3. In a second experiment, bovine corneal endothelial cells were used to study the stimulatory effects of proNGF and β-NGF on the migration of these cells. In a Boyden Chamber, corneal endothelial cells were stimulated with proNGF, β-NGF, or a test medium negative control. Cellular migration was determined after a period of 4 hours.

ProNGF (10^{-6} M) had significantly greater efficacy than β-NGF (10^{-6} M) in inducing bovine epithelial cell migration, as compared to the negative control.

Based on these results, it was concluded that proNGF itself acts as an active ingredient. In support of this conclusion, it is noted that if proNGF exhibited its activity only after cellular cleavage of the proNGF into β-NGF, proNGF could not have greater activity than β-NGF as observed in these experiments.



4. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted,

Date: 22. 12. 2005

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The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor.

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Abstract

Nerve growth factor (NGF) promotes neuronal survival and differentiation and stimulates neurite outgrowth. NGF is synthesized as a precursor, proNGF, which undergoes post-translational processing to generate mature beta-NGF. It has been assumed that, in vivo, NGF is largely processed into the mature form and that mature NGF accounts for the biological activity. However, we recently showed that proNGF is abundant in CNS tissues whereas mature NGF is undetectable, suggesting that proNGF has biological functions beyond its role as a precursor. To determine whether proNGF exhibits biological activity, we mutagenized the precursor-processing site and expressed unprocessed, cleavage-resistant proNGF protein in insect cells. Survival and neurite outgrowth assays on murine superior cervical ganglion neurons and PC12 cells indicated that proNGF exhibits neurotrophic activity similar to mature 2.5S NGF, but is approximately fivefold less active. ProNGF binds to the high-affinity receptor, TrkA, as determined by cross-linking to PC12 cells, and is also slightly less active than mature NGF in promoting phosphorylation of TrkA and its downstream signaling effectors, Erk1/2, in PC12 and NIH3T3-TrkA cells. These data, coupled with our previous report that proNGF is the major form of NGF in the CNS, suggest that proNGF could be responsible for much of the biological activity normally attributed to mature NGF in vivo.

PMID: 15086515 [PubMed - indexed for MEDLINE]

by reaction j producing (consuming) metabolite i by:

$$s'_{ij} = |s_{ij}|v_j$$

Random uptake conditions

We choose randomly X% (where $X = 10$, 50 or 80) of the 89 potential input substrates that *E. coli* consumes in addition to the minimal uptake basis. For each of the transport reactions, we set the uptake rate to 20 mmol per gram of dry weight per hour. As there are a very large number of possible combinations of the selected input substrates, we repeat this process 5,000 times and average over each realization.

The hit-and-run method

We select a set of basis vectors spanning the solution space using singular-value decomposition. Because the reaction fluxes must be positive, the 'bouncer' is constrained to the part of the solution space that intersects the positive orthant. We constrain the bouncer within a hypersphere of radius R_{max} and outside a hypersphere of radius $R_{min} < R_{max}$ where we find that the sampling results are independent of the choices of R_{min} and R_{max}. Starting from a random initial point inside the positive flux cone in a randomly chosen direction, the bouncer travels deterministically a distance d between sample points. Each sample point, corresponding to a solution vector where the components are the individual fluxes, is normalized by projection onto the unit sphere. After every bth bounce off the internal walls of the flux cone, the direction of the bouncer is randomized.

High-flux backbone

For each metabolite we keep only the reactions with the largest flux that produces and consumes the metabolite. Metabolites that are not produced (consumed) are discounted. Subsequently, a directed link is introduced between two metabolites A and B if (1) A is a substrate of the most active reaction producing B, and (2) B is a product of the maximal reaction consuming A. We consider only metabolites that are connected to at least one other metabolite after steps (1) and (2). For clarity, we remove P_i, PP_i and ADP. Further details and figures are provided in the Supplementary Information.

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Competing interests statement The authors declare that they have no competing financial interests.

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Sortilin is essential for proNGF-induced neuronal cell death

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Sortilin[1] (~95 kDa) is a member of the recently discovered family of Vps10p-domain receptors[2,3], and is expressed in a variety of tissues, notably brain, spinal cord and muscle. It acts as a receptor for neurotensin[4,5], but predominates in regions of the nervous system that neither synthesize nor respond to this neuropeptide[6], suggesting that sortilin has additional roles. Sortilin is expressed during embryogenesis[7] in areas where nerve growth factor (NGF) and its precursor, proNGF, have well-characterized effects[8,9]. These neurotrophins can be released by neuronal tissues[8,9], and they regulate neuronal development through cell survival and cell death signalling. NGF regulates cell survival and cell death via binding to two different receptors, TrkA and p75^{NTR} (ref. 10). In contrast, proNGF selectively induces apoptosis through p75^{NTR} but not TrkA[11]. However, not all p75^{NTR}-expressing cells respond to proNGF, suggesting that additional membrane proteins are required for the induction of cell death. Here we report that proNGF creates a signalling complex by simultaneously binding to p75^{NTR} and sortilin. Thus sortilin acts as a co-receptor and molecular switch governing the p75^{NTR}-mediated pro-apoptotic signal induced by proNGF.

Binding of NGF was examined by surface-plasmon resonance (SPR). As demonstrated in Fig. 1a, sortilin bound mature NGF with moderate affinity (dissociation constant (K_d) ~90 nM). In contrast, the affinity of NGF for p75^{NTR} and TrkA was high (K_d 1–2 nM), in accordance with previous studies in cells[11–13]. As the NGF precursor (proNGF) may escape intracellular processing and be released extracellularly, we next examined binding of proNGF[8,11,14,15]. Whereas lack of processing reduces the affinity of proNGF for p75^{NTR} and TrkA (K_d ~15–20 nM), it results in a much higher affinity (K_d ~5 nM) for sortilin (Fig. 1a). This is surprising because

proNGF has been reported to interact with cellular $p75^{NTR}$, but not TrkA, with a higher affinity (K_d ~0.2 nM) than mature NGF, and to selectively induce $p75^{NTR}$-dependent apoptosis in neurons, smooth muscle cells and oligodendrocytes[8,11]. Our data may reflect the participation of a $p75^{NTR}$ co-receptor, and this receptor might be sortilin (see below).

To examine the structural basis of proNGF binding, we produced the pro domain of proNGF as a glutathione *S*-transferase fusion protein (GST–pro) (Fig. 1b). The GST–pro protein bound to sortilin with an affinity very similar to that of proNGF (K_d ~8 nM), but not to $p75^{NTR}$ or TrkA (Fig. 1a). Additional experiments further demonstrated that binding of proNGF to sortilin was inhibited markedly (>75%) by neurotensin, and was almost abolished in the presence of GST–pro (Fig. 1c). Thus, the pro domain constitutes the structural basis for the high-affinity binding between proNGF and sortilin.

We next assessed binding of proNGF to cellular sortilin. We transfected 293 cells without endogenous sortilin with the receptor constructs indicated in Fig. 2a, and evaluated binding of proNGF at 37 °C. Control cells demonstrated no binding or uptake of ligand, whereas cells expressing wild-type sortilin exhibited significant endocytosis of proNGF (Fig. 2b), but not of mature NGF (Fig. 2c). The observed uptake was hampered strongly in the presence of excess neurotensin or GST–pro (data not shown). Furthermore, transfectants expressing a mutant sortilin protein (sortilin(mut)) that accumulates on the plasma membrane owing to disrupted motifs for endocytosis[16], displayed intense surface labelling with proNGF, but little uptake.

Binding and uptake of proNGF was also investigated in cells transfected with TrkA and $p75^{NTR}$, and in cells expressing each of the two receptors in combination with sortilin (Fig. 2b). TrkA transfectants exhibited a very modest uptake of proNGF, and on co-transfection with sortilin, endocytosis of proNGF was comparable to that observed in cells expressing sortilin alone. In contrast, uptake of mature NGF was efficient in TrkA-expressing cells and was unaffected by co-transfection with sortilin (Fig. 2c). In $p75^{NTR}$ transfectants, proNGF as well as mature NGF was almost exclusively found on the plasma membrane, indicating a slow or insignificant endocytosis (Fig. 2b, c) consistent with prior observations[17,18]. However, coexpression of $p75^{NTR}$ with sortilin re-established uptake of proNGF, and coexpression with sortilin(mut), as well as with wild-type sortilin, induced a significant increase in surface-associated ligand, suggesting a synergistic rather than a simple additive effect of sortilin and $p75^{NTR}$ coexpression.

The findings demonstrate that sortilin exhibits negligible binding of NGF, but also that it conveys a significantly higher capacity for uptake of proNGF than either of the two established receptors (that is, $p75^{NTR}$ and TrkA). Moreover, results in double transfectants suggest that sortilin and $p75^{NTR}$ cooperate to promote proNGF binding.

To characterize the molecular mechanisms underlying the putative cooperativity between $p75^{NTR}$ and sortilin, affinity crosslinking was performed. Crosslinking of ^{125}I-labelled proNGF to $p75^{NTR}$ and sortilin double transfectants produced labelled complexes of ~110, ~140 and ~240 kDa (Fig. 3a, lane 1), but was unproductive in single transfectants, suggesting that coexpression of sortilin and $p75^{NTR}$ is required for efficient binding at subnanomolar concentrations of proNGF (Fig. 3a, lanes 5–6). Furthermore, immunoprecipitation with receptor antisera established that both sortilin and $p75^{NTR}$ were components of the crosslinked adducts (Fig. 3a, lanes 7–8). Similar experiments performed on cells coexpressing $p75^{NTR}$ and sortilin(mut), which has a higher surface expression than wild-type sortilin[16], resulted in a quantitative increase in crosslinked complexes (data not shown). Finally, generation of the crosslinking adducts was markedly reduced in the presence of unlabelled proNGF, neurotensin or GST–pro, implying that sortilin, as well as the NGF pro domain, is critical to complex formation (Fig. 3a, lanes 2–4).

We conclude that the ~240 kDa adduct probably represents a heterotrimeric complex comprising proNGF, sortilin and $p75^{NTR}$, whereas the ~110 kDa and ~140 kDa species constitute proNGF in association with a single receptor. Expression of both receptors is required for efficient binding of proNGF, and our results support a model in which the pro domain and the 'mature' part of proNGF simultaneously engage sortilin and $p75^{NTR}$, respectively.

Corresponding experiments established that proNGF does not form stable complexes with TrkA (Fig. 3b). In fact, crosslinking with proNGF using cells expressing all three receptors (TrkA, $p75^{NTR}$ and sortilin) resulted in ~110, ~140 and ~240 kDa adducts that could be precipitated with anti-sortilin (data not shown) and anti-$p75^{NTR}$ antibodies but not with TrkA-specific antiserum. Thus, proNGF discriminates between TrkA and $p75^{NTR}$ in cells that express both receptors in combination with sortilin.

In accordance with previous reports[12,13], crosslinking using mature ^{125}I-labelled NGF yielded complexes with both $p75^{NTR}$ (~90 and ~180 kDa) and TrkA (~160 kDa) (Fig. 3c). However,

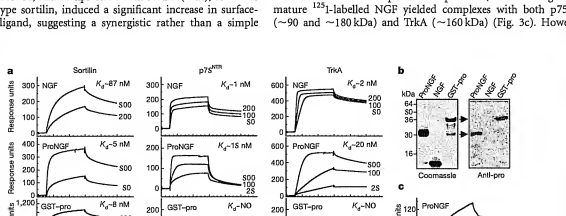

Figure 1 SPR analysis of ligand binding. **a**, Binding of 20–1,000 nM NGF, proNGF and GST–pro to immobilized sortilin (51 fmol mm^{-2}), $p75^{NTR}$ (91 fmol mm^{-2}) and TrkA (66 fmol mm^{-2}). Calculated K_d values are indicated. **b**, SDS–PAGE analysis of ligands used in **a**. A Coomassie-stained gel (left panel) and a western blot (anti-pro domain antibody; right panel) are shown. **c**, Binding of proNGF (25 nM) to sortilin (66 fmol mm^{-2}) in the absence or presence of 10 μM neurotensin (dashed line) and 5 μM GST–pro (dotted line). The SPR response obtained for the inhibitors alone has been subtracted.

no additional crosslinked complexes were observed when either of the two was coexpressed with sortilin, and sortilin single transfectants did not bind NGF. These results indicate that sortilin neither interacts with mature NGF nor is part of a complex formed on binding of mature NGF to $p75^{NTR}$ or TrkA.

We next examined whether sortilin and $p75^{NTR}$ physically associate on the cell membrane. Cells expressing both receptors were biolabelled and incubated in the absence or presence of proNGF, followed by treatment with a membrane-impermeable reducible crosslinker, lysis and immunoprecipitation using anti-$p75^{NTR}$ antibodies. Sortilin could be crosslinked directly to $p75^{NTR}$ (Fig. 3d). However, in the presence of proNGF, the relative amount of crosslinked and co-precipitated sortilin increased by about fivefold (3.9% to 18.4%). Equilibrium binding studies were then designed to determine whether $p75^{NTR}$ and sortilin coexpression might influence the specificity and affinity of ligand binding. As demonstrated in Fig. 3e, ^{125}I-labelled NGF bound to cells coexpressing sortilin and $p75^{NTR}$ with an estimated K_d of ~1.0 nM. This agrees with previous results[12] obtained in $p75^{NTR}$-expressing cells, and as sortilin single transfectants did not bind mature NGF (data not shown), the data indicate that mature NGF binds strictly to $p75^{NTR}$. In contrast, similar experiments with ^{125}I-labelled proNGF further indicated that sortilin and $p75^{NTR}$ cooperate in proNGF binding. Thus, cells expressing a single receptor type—either sortilin or $p75^{NTR}$—did not bind ^{125}I-labelled proNGF (data not shown), whereas cells coexpressing these receptors did. Scatchard analysis (Fig. 3e) suggested fewer binding sites for proNGF than for NGF in the double transfectants, but also a higher affinity for proNGF (K_d ~160 pM) that could not be accounted for by binding to any single receptor. Accordingly, A875 cells, which bind proNGF with high affinity[11], express high levels of endogenous sortilin and $p75^{NTR}$

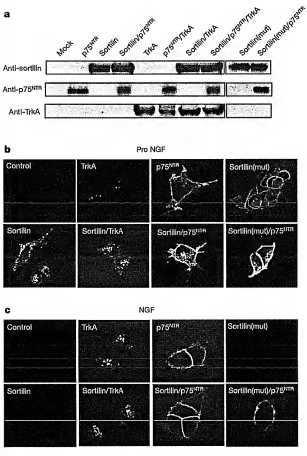

Figure 2 Binding and uptake of proNGF and NGF in 293 cells. **a**, Western blot showing the level of receptor expression in the transfected 293 cells. **b**, **c**, Untransfected cells (control) and cells transfected with the indicated receptors were incubated (37 °C, 45 min) with 50 nM proNGF (**b**) or NGF (**c**) before fixation and staining with anti-NGF antibodies.

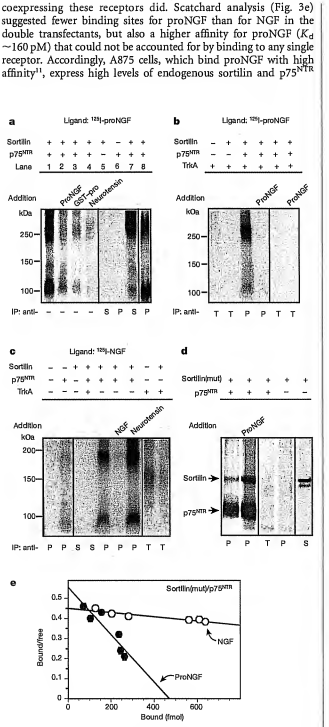

Figure 3 ProNGF-induced formation of heterotrimeric complexes comprising sortilin and $p75^{NTR}$. **a**–**c**, Crosslinking of ^{125}I-labelled proNGF (**a**, **b**) or ^{125}I-labelled NGF (**c**) to transfected 293 cells in the presence and absence of excess proNGF, NGF, GST–pro or neurotensin. Crude lysates (−) and immunoprecipitated proteins were subjected to SDS–PAGE and labelled bands were visualized by autoradiography. Antibodies used for immunoprecipitation (IP) of sortilin (S), $p75^{NTR}$ (P) and TrkA (T) are indicated. **d**, Biolabelled cells overexpressing sortilin(mut) and $p75^{NTR}$ were incubated with and without proNGF (25 nM) and treated with a reducible crosslinker. Autoradiographic bands resulting from reducing SDS–PAGE of immunoprecipitates are shown. **e**, Scatchard plot showing binding of radiolabelled NGF (open circles) and proNGF (closed circles) to cells expressing sortilin(mut) and $p75^{NTR}$.

(Fig. 4a). The results support a model in which proNGF binds to and promotes the formation of a multi-component receptor complex comprising both sortilin and $p75^{NTR}$.

ProNGF is more efficient than NGF in inducing apoptosis in superior cervical ganglion (SCG) neurons, vascular smooth muscle (SM-11) cells and oligodendrocytes, and in promoting chemotaxis and ligand binding in A875 melanoma cells[8,11,19]. These cell types all express significant levels of sortilin and $p75^{NTR}$ (Fig. 4a). We found that uptake of proNGF in dissociated SCG cultures was inhibited by the GST–pro protein (data not shown), which selectively inhibits binding to sortilin. Moreover, crosslinking of ^{125}I-labelled proNGF to SM-11 cells resulted in labelled adducts of ~110, ~140 and ~240 kDa, similar to those obtained in the $p75^{NTR}$ and sortilin double transfectants (Fig. 4b). These findings suggest that the biological effects of proNGF require coexpression of sortilin and $p75^{NTR}$. To assess directly whether binding of proNGF to sortilin regulates biological action, the ability of GST–pro and neurotensin to impair proNGF actions was evaluated. In order to minimize conversion of proNGF into mature NGF, which might introduce a bias by facilitating survival in TrkA-positive cells, a furin-resistant mutant of proNGF[11] was used in all subsequent experiments, unless otherwise stated. In SM-11 cells co-expressing $p75^{NTR}$ and sortilin, furin-resistant proNGF was more effective than mature NGF in inducing cell death as assessed by a TdT-mediated dUTP nick end labelling (TUNEL) assay (Fig. 4c). In addition, wild-type proNGF induced apoptosis as effectively as furin-resistant proNGF (data not shown). Co-incubation of proNGF with an excess of neurotensin, or with excess GST–pro, but not GST alone, impaired the induction of cell death and apoptosis by more than 90% (Fig. 4c). Similar findings were obtained in cultured SCG neurons expressing sortilin and $p75^{NTR}$ as well as TrkA. Thus, both GST–pro and neurotensin significantly reduced proNGF-induced apoptosis in SCG neurons,

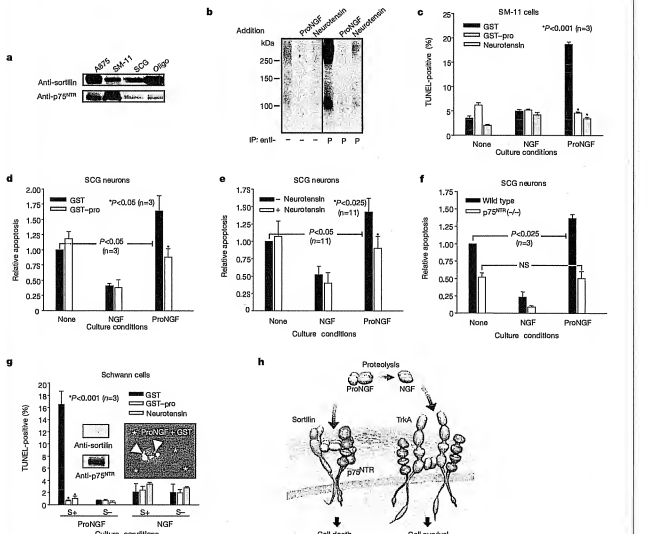

Figure 4 Sortilin is required for the pro-apoptotic action of proNGF. **a**, Receptor expression in proNGF-responsive cell types. **b**, Crosslinking of ^{125}I-labelled proNGF to SM-11 cells and inhibition by unlabelled competitors. **c**–**e**, ProNGF-induced apoptosis (cell type indicated) and its inhibition by GST, GST–pro and neurotensin. The number of apoptotic and total cells counted per condition was 100/~300 (**d** and **f**) and 300/~1,100 (**e**). All values are normalized to apoptosis in the absence of any additions. **f**, Killing of SCG neurons from wild-type and $p75^{NTR}$ knockout mice. **g**, ProNGF-induced apoptosis of Schwann cells transiently transfected with sortilin. Columns indicate per cent of TUNEL-positive cells among cells that express (S+) or lack (S−) sortilin. Left inset shows western blot of Schwann cell lysate; right inset shows apoptotic nuclei (TUNEL-positive, green) in transfectants expressing sortilin (red staining). Asterisk indicates an untransfected cell. **h**, Schematic model of receptor–complex formation.

whereas neither affected neuronal survival in response to mature NGF or on NGF withdrawal (Fig. 4d, e). As shown in Fig. 4f, $p75^{NTR}$-deficient neurons from $p75^{NTR}$ knockout mice[20] that express sortilin in the absence of $p75^{NTR}$ (data not shown) exhibit NGF-dependent survival, but are resistant to proNGF-induced killing. A similar resistance to proNGF was seen in Schwann cells expressing $p75^{NTR}$ but not sortilin (Fig. 4g); however, after transfection with sortilin, Schwann cells became sensitive to proNGF-induced apoptosis. Approximately 95% of the cells that expressed sortilin (~18% of total) were TUNEL positive, and among all TUNEL-positive cells ~96% expressed both sortilin and $p75^{NTR}$. This sensitivity to proNGF-induced killing was reversed by co-incubation with GST–pro (Fig. 4g) and neurotensin, which blocked binding of proNGF to sortilin. It follows that both receptors are obligate for the induction of proNGF-mediated cell death, whereas sortilin expression has no impact on NGF responsiveness under these circumstances.

We conclude that proNGF targets and promotes formation of a signalling complex comprising endogenous sortilin and $p75^{NTR}$, and that both receptors are required for proNGF-mediated apoptosis. In contrast, mature NGF preferentially binds $p75^{NTR}$ and/or TrkA, with sortilin having little or no bearing on NGF-initiated signalling.

Our study indicates that the neurotrophins use not two but three distinct receptor classes to dictate and regulate opposing biological responses of survival and death. We identify sortilin as a biologically important neurotrophin receptor that targets the pro domain of proNGF with high affinity. The present data suggest that sortilin is a required component for transmitting proNGF-dependent death signals via $p75^{NTR}$. Together with $p75^{NTR}$, sortilin facilitates the formation of a composite high-affinity binding site for proNGF (Fig. 4h). Thus, sortilin serves as a co-receptor and molecular switch, enabling neurons expressing Trk and $p75^{NTR}$ to respond to a pro-neurotrophin and to initiate pro-apoptotic rather than pro-survival actions. In the absence of sortilin, regulated activity of extracellular proteases may cleave proNGF to mature NGF[11], promoting Trk-mediated survival signals (Fig. 4h). In conclusion, NGF-induced neuronal survival and death is far more complicated than previously appreciated, as it depends on an intricate balance between proNGF and mature NGF, as well as on the spatial and temporal expression of three distinct receptors: TrkA, $p75^{NTR}$ and sortilin. As sortilin is but one member of the Vps10p-domain receptor family expressed in the nervous system, future studies should show whether other pro-neurotrophins use related Vps10p-containing receptors to switch biological responsivity to neurotrophin isoforms. □

Methods

Recombinant proteins and radiolabelling

Human proNGF and mature NGF generated in *Escherichia coli*[21] were a gift from Scil Proteins GmbH. Radioiodinated ligands[20,23] (~3,000 d.p.m. $fmol^{-1}$) were used within 48 h of iodination and their integrity and bioactivity was assayed by SDS–polyacrylamide gel electrophoresis (PAGE), PC12 cell neuritogenesis (NGF) and apoptosis of $p75^{NTR}$-expressing vascular smooth muscle cells (proNGF). Mature NGF and furin-resistant mouse proNGF were purified from media of transfected 293 cells[18]. The NGF pro domain (amino acids E19 to R121) was expressed in *E. coli* as a GST fusion protein and purified on glutathione–agarose beads. The luminal domain of sortilin was expressed and purified as described[9]. $p75^{NTR}$-Fc and TrkA-Fc (fusion proteins) were from R&D Systems.

SPR analysis, equilibrium binding and western blotting

The SPR analysis was performed essentially as described[24]. The receptors were immobilized (at 10–15 $\mu g\,ml^{-1}$) on a CM5 chip and remaining coupling sites were blocked with 1 M ethanolamine. Sample and running buffer was 10 mM HEPES, 150 mM $(NH_4)_2SO_4$, 1.5 mM $CaCl_2$, 1 mM EGTA, 0.005% Tween-20 pH 7.4. After each analytic cycle the sensor chip was regenerated in a 10 mM glycine-HCl buffer. The SPR signal was expressed in relative response units (RU); that is, the response obtained in a control flow channel was subtracted. Kinetic parameters were determined using BIAevaluation 3.1 software. Equilibrium-binding studies were performed as described[25]. In brief, the cells ($2 \times 10^6\ ml^{-1}$) were incubated (4 °C, 40 min) with radioiodonated NGF or proNGF ($2–20 \times 10^{-12}$ M) in the presence or absence of a 500-fold molar excess of NGF or neurotensin, respectively. Bound ligand was then separated from free ligand by centrifugation through cold serum. Mean values of triplicates (from two independent experiments) were evaluated using the PRISM program.

Western blotting, after reducing SDS–PAGE, was performed using a rabbit antibody (1:1,000) directed against the pro domain of NGF (amino acids 23–81) of human NGF and horseradish-peroxidase-conjugated swine anti-rabbit immunoglobulin (Amersham Biosciences).

Transfected cell lines

Parental 293 cells and transfectants expressing $p75^{NTR}$ or TrkA[18] were transfected with wild-type sortilin[9] or the sortilin(mut) variant impaired in endocytosis (alanine substituted for Y14, L17, L51 and L52 in the cytoplasmic tail)[18], and selected using zeocin. Primary rat Schwann cells[26], plated on polylysine-coated plates, were transfected with pcDNA wild-type sortilin[9] or pcDNA alone using calcium phosphate.

Crosslinking, biolabelling and immunoprecipitation

Cells ($2 \times 10^6\ ml^{-1}$) were incubated (4 °C, 2 h) with radioiodinated proNGF or NGF (400 pM), in the absence or presence of 100 nM unlabelled proNGF, 40 μM neurotensin or 200 nM GST or GST–pro, followed by crosslinking (15 min) with 4 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and 25 mM DSS (Pierce). Washed cells were subsequently lysed in 1% Nonidet P40 buffer containing protease inhibitors and precipitations were performed using anti-$p75^{NTR}$ and anti-TrkA antisera[18], and anti-sortilin antibody[9].

Transfected cells were biolabelled (3–4 h) with L-[^{35}S]cysteine and L-[^{35}S]methionine, then incubated (20 °C, 2 h) with or without 25 nM proNGF and, finally, treated (30 min) with 5 mM of the reducible crosslinker DTSSP (Pierce) before lysis in 1% Triton-X100 buffer containing protease inhibitors[9,18]. Immunoprecipitation was performed using rabbit anti-$p75^{NTR}$ (number 9993), anti-Trk (Santa Cruz) and anti-sortilin[9]. All precipitated proteins were analysed by reducing SDS–PAGE and were detected by autoradiography.

Induction of apoptosis in various cells

A vascular smooth muscle cell line expressing human $p75^{NTR}$ but not TrkA[27] was incubated (16 h) with 2 $ng\,ml^{-1}$ of mature NGF or furin-resistant proNGF[18] in the presence of 50 nM GST or GST–pro, or 40 μM neurotensin. After fixation, cells were fixed, incubated with 4,6-diamidino-2-phenylindole (DAPI) and subjected to TUNEL analysis (Roche Molecular Biochemicals). Results represent the mean value of three independent experiments performed in triplicate. At least 300 cells per condition were counted.

Dissociated P0–P1 rat SCG neurons[28] or mouse SCG neurons obtained from $p75^{NTR}$ knockout mice[20] or wild-type littermates were plated on collagen-coated slides and maintained for 5 days in 50 $ng\,ml^{-1}$ NGF before use. Replicate cultures were rinsed five times with NGF-free medium and treated with or without the given additives, and fixed. After 36 h SCG cultures were processed for TUNEL analysis and counterstained with anti-neuronal-specific β-tubulin (Tuj1, Covance)[11]. TUNEL-positive neurons were scored blindly by the observer and at least 100 cells were counted for each culture condition.

Transfected Schwann cells were plated on 8-well slides (NUNC) at 20,000 cells per well. At 48 h after transfection, cells were treated (18 h) with 5 $ng\,ml^{-1}$ mature NGF, purified recombinant cleavage-resistant proNGF or diluent alone. After fixation, the cells were stained using mouse anti-sortilin antibody (Transduction Biolabs, anti-NTR3 612101) and rhodamine goat anti-mouse IgG followed by DAPI incubation, and then subjected to TUNEL analysis (Roche Molecular Biochemicals). At least 1,000 cells per condition were counted in a blinded manner, and results are representative of three independent experiments. Where appropriate, statistical significance was determined by Student's *t*-test.

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The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys

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Host cell barriers to the early phase of immunodeficiency virus replication explain the current distribution of these viruses among human and non-human primate species[1–4]. Human immunodeficiency virus type 1 (HIV-1), the cause of acquired immunodeficiency syndrome (AIDS) in humans, efficiently enters the cells of Old World monkeys but encounters a block before reverse transcription[5–8]. This species-specific restriction acts on the incoming HIV-1 capsid[5–7] and is mediated by a dominant repressive factor[7–9]. Here we identify TRIM5α, a component of cytoplasmic bodies, as the blocking factor. HIV-1 infection is restricted more efficiently by rhesus monkey TRIM5α than by human TRIM5α. The simian immunodeficiency virus, which naturally infects Old World monkeys[10], is less susceptible to the TRIM5α-mediated block than is HIV-1, and this difference in susceptibility is due to the viral capsid. The early block to HIV-1 infection in monkey cells is relieved by interference with TRIM5α expression. Our studies identify TRIM5α as a species-specific mediator of innate cellular resistance to HIV-1 and reveal host cell components that modulate the uncoating of a retroviral capsid.

Recombinant HIV-1 expressing green fluorescent protein and pseudotyped with the vesicular stomatitis virus (VSV) G glycoprotein (denoted HIV-1–GFP) can efficiently infect the cells of many mammalian species including humans, but not those of Old World monkeys[4–9]. Here we used a murine leukaemia virus vector to transduce human HeLa cells, which are susceptible to HIV-1–GFP infection, with a complementary DNA library prepared from primary rhesus monkey lung fibroblasts (PRL cells). Two independent HeLa clones resistant to HIV-1–GFP infection, but susceptible to infection with recombinant simian immunodeficiency virus (SIV–GFP) or murine leukaemia virus (MLV–GFP), were identified in a screen (Methods).

The only monkey cDNA insert common to both HIV-1–GFP-resistant clones was predicted to encode TRIM5α, a member of the tripartite motif (TRIM) family of proteins containing RING domains, B-boxes and coiled coils[11–13]. TRIM5α also contains a carboxy-terminal B30.2 (SPRY) domain not found in the other TRIM5 isoforms (ref. 13 and Fig. 1a). The natural functions of TRIM5α, or of the cytoplasmic bodies in which the TRIM5 proteins localize[13,14], are unknown. One TRIM5 isoform has been shown to have ubiquitin ligase activity typical of RING-containing proteins[14]. TRIM5 proteins are expressed constitutively in many tissues[13], consistent with the pattern of expression expected for the HIV-1-blocking factor in monkeys[4].

HeLa cells stably expressing rhesus monkey TRIM5α (TRIM5α$_{rh}$) and control HeLa cells containing empty vector were incubated with different amounts of recombinant HIV-1–GFP, SIV–GFP and MLV–GFP. Expression of TRIM5α$_{rh}$ resulted in a marked inhibition of infection by HIV-1–GFP, whereas MLV–GFP infected control and TRIM5α$_{rh}$-expressing HeLa cells equivalently (Fig. 1b, c). TRIM5α$_{rh}$ inhibited infection by SIV–GFP less efficiently than that by HIV-1–GFP (Fig. 1c). Stable TRIM5α$_{rh}$ expression also inhibited the replication of infectious HIV-1 in HeLa-CD4 cells, which express the receptors for HIV-1 (ref. 15 and Fig. 1d). The replication of a simian–human immunodeficiency virus (SHIV) chimaera, which contains core proteins (including the capsid protein) of SIV$_{mac}$ (ref. 16), was not inhibited in these TRIM5α$_{rh}$-expressing cells. When the infections were done with eightfold more HIV-1 and SHIV, similar results were obtained (Supplementary Information). We conclude that expression of TRIM5α$_{rh}$ specifically and efficiently blocks infection by HIV-1, and exerts a slight inhibitory effect on infection by SIV$_{mac}$.

To investigate the viral target of the TRIM5α$_{rh}$-mediated restriction, HeLa cells expressing TRIM5α$_{rh}$ or control HeLa cells were incubated with recombinant HIV-1–GFP, SIV–GFP, SIV(HCA-p2)–GFP or HIV(SCA)–GFP. SIV(HCA-p2)–GFP is identical to SIV–GFP, except that the SIV capsid and adjacent p2 sequences have been replaced by those of HIV-1 (ref. 17), and SIV(HCA-p2)–GFP has been shown to be susceptible to the block in Old World monkey cells[5,7]. HIV(SCA)–GFP is identical to HIV-1–GFP, except that most of the capsid protein has been replaced by that of SIV[5], and HIV(SCA)–GFP has been shown to be less susceptible than HIV-1 to the block in Old World monkey cells[5]. We found that HIV-1-GFP and SIV(HCA-p2)–GFP infections were restricted to the same extent in TRIM5α$_{rh}$-expressing HeLa cells, whereas infec-

X. Related Proceedings Appendix

None.